CHARACTERIZING CHANGES IN THE OREXIN SYSTEM IN MODELS OF NEUROPSYCHIATRIC DISEASE

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Bachelor of Psychology (Hons)

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DECLARATION

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Erin Jane Campbell

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropin-releasing hormone
BLA	Basolateral amygdala
CeL	Central nucleus of the amygdala, lateral subdivision
CeM	Central nucleus of the amygdala, medial subdivision
CNO	Clozapine-N-oxide
CPP	Conditioned place preference
CRF	Corticotropin-releasing factor
CS	Conditioned stimulus
CTb	Cholera toxin subunit B
D1R	Dopamine D1 receptors
DMH	Dorsomedial hypothalamus
DORA	Dual orexin receptor antagonist
DREADD	Designer receptors exclusively activated by designer drugs
ELS	Early life stress
EPM	Elevated plus maze
FR1	Fixed-ratio 1 schedule of reinforcement
FR3	Fixed-ratio 3 schedule of reinforcement
HPA	Hypothalamic pituitary adrenal axis
IR	Immunoreactive cells
KORD	Kappa opioid-receptor based DREADD
LH	Lateral hypothalamus
LPS	Lipopolysaccharide
LTP	Long-term potentiation

MCH	Melanin-concentrating hormone
MeA	Medial nucleus of the amygdala
NAc	Nucleus accumbens
OF	Open field
OxR1/2	Orexin receptors 1/2
PAG	Periaqueductal gray
PFA	Perifornical area of the hypothalamus
PND	Postnatal day
PR	Progressive ratio schedule of reinforcement
PVN	Paraventricular nucleus of the hypothalamus
PVT	Paraventricular thalamus
SORA	Single orexin receptor antagonist
TH	Tyrosine hydroxylase
VGAT	Vesicular GABA transporter
VGLUT2	Vesicular glutamate transporter 2
vmPFC	Ventral medial prefrontal cortex
vSub	Ventral subiculum
VTA	Ventral tegmental area
WKY	Wistar-Kyoto rat

ABSTRACT

Hypothalamic dysfunction is a key feature of several neuropsychiatric disease states, including those involving reward-related deficits such as depression. Interestingly, exposure to early life adverse events, such as childhood trauma, typically precipitates the development of depression in adulthood. Most preclinical research regarding early life stress (ELS) has focussed on neuroendocrine cell populations. However, the lateral hypothalamus (LH) is also known to influence autonomic, neuroendocrine and behavioural responses to stress but its role in depressive-like behaviours such as low motivational drive is less well studied and was therefore the focus of this thesis. Within the LH there are several neuronal populations expressing GABA, and glutamate as well as neuropeptides such as orexin (hypocretin) and melanin-concentrating hormone (MCH). Notably, orexin has been implicated in reward-seeking pathways focussing on drug (cocaine) and natural reward-seeking behaviour (high fat and sugar foods). The overarching aim of my thesis was to assess the role of the hypothalamic orexin system in models of ELS in precipitating behaviours relevant to neuropsychiatric disease states such as suppressed reward-seeking behaviour.

Using behavioural techniques and neural activity mapping, I first demonstrated that our model of ELS (maternal separation) suppressed motivated arousal in response to restraint stress in adulthood; an effect that was associated with a reduction in the percentage of Fos-positive orexin cells. Interestingly, voluntary wheel-running reversed both behavioural and neural deficits observed after ELS. A question that arose from these studies was whether these deficits in reward-seeking were produced by specific circuits within the LH. Further, given the difficulty in overcoming deficits in motivational drive, and in many cases an inability to exercise to beneficial levels in depressed individuals, I wanted to assess the feasibility of manipulating LH circuits pharmacologically to reverse ELS-induced deficits.

To achieve my second aim, I applied for and received a prestigious travel grant from the Hunter Medical Research Institute to study at the National

Institute on Drug Abuse (NIDA), Baltimore, USA to learn a novel technique termed chemogenetics . Professor Yavin Shaham's lab at NIDA had recently employed chemogenetics to isolate specific circuits in the LH. At NIDA I used a dual-virus approach to inhibit ventral subiculum to nucleus acumbens shell projection neurons. Selective inhibition of this pathway decreased context-induced relapse to alcohol seeking in rats. Upon returning to the University of Newcastle, I implemented these chemogenetic approaches to my model of ELS. I showed that chemogenetic activation of the LH reversed ELS-induced deficits in the motivation to lever press for a sucrose reward. Interestingly, this recovery of sucrose responding was associated with increases in the number of Fos-positive orexin neurons; but also a significant number of non-orexin, putative GABAergic neurons were recruited .

In the final chapter of my thesis, I sought to determine if the orexin system was also altered following another type of ELS, exposure to an early life immune challenge lipopolysaccharide (LPS). LPS is an endotoxin bacterial cell wall product modelling the prevalence of bacterial immune stimuli in the perinatal human environment. Interestingly, early life LPS followed by formalin-induced inflammatory pain in adulthood resulted in an increase in the number of Fos-positive orexin cells.

Together the data from my thesis indicate that that ELS rewires LH-orexin circuitry, as well as other LH projection pathways, and that these changes manifest as inappropriate behavioural responses to psychological and physical challenges in later life. Interestingly, exercise and chemogenetic activation of the LH is able to reverse the behavioural deficits produced by ELS. These data are important clinically because they suggest that capacity remains in LH circuitry, to overcome ELS-induced deficits in motivational drive.

INTRODUCTION

Orexin antagonists for neuropsychiatric disease: progress and potential pitfalls.

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Orexin antagonists for neuropsychiatric disease: progress and potential pitfalls

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The tight regulation of sleep/wake states is critical for mental and physiological wellbeing. For example, dysregulation of sleep/wake systems predisposes individuals to metabolic disorders such as obesity and psychiatric problems, including depression. Contributing to this understanding, the last decade has seen significant advances in our appreciation of the complex interactions between brain systems that control the transition between sleep and wake states. Pivotal to our increased understanding of this pathway was the description of a group of neurons in the lateral hypothalamus (LH) that express the neuropeptides orexin A and B (hypocretin, Hcrt-1 and Hcrt-2). Orexin neurons were quickly placed at center stage with the demonstration that loss of normal orexin function is associated with the development of narcolepsy—a condition in which sufferers fail to maintain normal levels of daytime wakefulness. Since these initial seminal findings, much progress has been made in our understanding of the physiology and function of the orexin system. For example, the orexin system has been identified as a key modulator of autonomic and neuroendocrine function, arousal, reward and attention. Notably, studies in animals suggest that dysregulation of orexin function is associated with neuropsychiatric states such as addiction and mood disorders including depression and anxiety. This review discusses the progress associated with therapeutic attempts to restore orexin system function and treat neuropsychiatric conditions such as addiction, depression and anxiety. We also highlight potential pitfalls and challenges associated with targeting this system to treat these neuropsychiatric states.

Keywords: hypothalamus, orexin, stress, anxiety, depression, cocaine, reinstatement, reward seeking

OVERVIEW—THE OREXIN SYSTEM IN BRIEF

First described in 1996, the orexins are two neuropeptides expressed by a few thousand neurons within the perifornical area (PFA), the dorsomedial hypothalamus (DMH) and the lateral hypothalamus (LH) (de Lecea et al., 1998; Sakurai et al., 1998). The binding target of these ligands, termed orexin A and B, are two G-protein coupled receptors OxR1 and OxR2 (de Lecea et al., 1998; Sakurai et al., 1998). Orexin A is non-selective for both OxR1 and OxR2 whereas orexin B is more selective for OxR2 (Sakurai et al., 1998; Ammoun et al., 2003). A key feature of this relatively small population of neurons is their widespread projections throughout the brain, including other hypothalamic nuclei, the midline paraventricular thalamus (PVT), brain stem nuclei and a number of structures involved in reward behavior including the ventral tegmental area (VTA) and nucleus accumbens shell (NACs) (Peyron et al., 1998). In these projection areas, the expression of orexin receptor subtypes is partially overlapping, however, some regions preferentially express one receptor subtype, presumably providing some degree of selectivity (certainly in terms of potential pharmacological selectivity of different target regions). For example, the prefrontal cortex predominantly expresses OxR1, whereas the nucleus accumbens (NAC) mainly expresses OxR2 (Marcus et al., 2001).

Consistent with the widespread projections of these neurons, orexins have been implicated in a number of physiological functions, including regulation of sleep (Chemelli et al., 1999), energy metabolism (Burdakov et al., 2005), arousal (Sutcliffe and de Lecea, 2002; Taheri et al., 2002), behavioral and neuroendocrine responses to stress (Ida et al., 2000; Furlong et al., 2009) and reward-seeking behavior (Boutrel et al., 2005; Harris et al., 2005; Lawrence et al., 2006; Marchant et al., 2012). The role of orexin in this diverse range of functions has been reviewed extensively elsewhere (Boutrel and de Lecea, 2008; Aston-Jones et al., 2010; Boutrel et al., 2010; Lawrence, 2010; James et al., 2012; Mahler et al., 2012). In this review, we will highlight new research focused on changes in the intra-hypothalamic LH-orexin circuitry induced by drugs of abuse and stress. We will also outline recent data highlighting the clinical potential of single and dual orexin receptor antagonists (SORAs and DORAs) for neuropsychiatric conditions including addiction, anxiety and depression. However, we also discuss recent findings indicating that several challenges must be overcome for the therapeutic value of SORAs and DORAs to be fully realized.

CELLULAR AND MOLECULAR EVIDENCE FOR TARGETING THE LH-OREXIN SYSTEM IN DRUG ADDICTION

Until recently, work on the relationship between the orexin system and addiction has been heavily focused on the antagonism of orexin actions in downstream projection areas (James et al., 2011; Brown et al., 2013; Mahler et al., 2013). At the same time, the issue of how drugs of addiction might alter the properties of orexin neurons has been largely overlooked. Interestingly, gene expression analysis has shown that the LH is highly transcriptionally responsive to addictive drugs (Ahmed et al., 2005). Ahmed et al. (2005) found that rats given extended access to cocaine displayed a profound increase in both pre- and post-synaptic markers of plasticity in the LH. Further, studies from the feeding literature suggest that excitatory inputs onto orexin neurons can undergo significant rewiring in response to food-deprivation and re-feeding. These results suggest that the LH orexin circuitry is likely to undergo experience-dependent neuroplasticity in response to cocaine treatment.

Given the above evidence, our group has undertaken a series of studies to assess how drugs might remodel LH orexin circuits. In these experiments, rats were exposed to 7 days of cocaine injections or allowed to self-administer cocaine for 14 days before excitatory synaptic transmission was assessed in LH slices. This work showed that both experimenter- and self-administered cocaine significantly increased excitatory drive in the LH through presynaptic mechanisms, assessed using mEPSCs frequency, amplitude and paired pulse ratio (Yeoh et al., 2012). Consistent with our electrophysiological findings, the number of putative excitatory but not inhibitory inputs onto PFA/LH cells were significantly increased, as measured by immunolabeling for vesicular glutamate transporter 2 (VGLUT2) or vesicular GABA transporter (VGAT; Yeoh et al., 2012). Importantly, a population of recorded neurons that were recovered with neurobiotin labeling and immunolabeled for orexin confirmed that these increases in excitatory drive occurred in orexin neurons.

Somewhat surprisingly, a recent study by a different group failed to find evidence of pre-synaptic plasticity in orexin neurons after 3 days of cocaine exposure (Rao et al., 2013). However, this regimen of cocaine exposure did promote long-term potentiation (LTP) at glutamate synapses onto orexin neurons, which persisted for more than 5 days post-withdrawal (Rao et al., 2013). The differences in the Rao et al. (2013) study and our own may in part be explained by differences in experimental procedures. In our study, we carried out both intraperitoneal (i.p) injections and cocaine self-administration in rats as compared to the Rao study, which employed a conditioned place preference (CPP) model whereby mice received only experimenter-administered cocaine injections. Animals that underwent i.p injections in our study received a slightly higher dose of cocaine for a longer period of time as compared to Rao and colleagues (15 mg/kg/7 days vs. 10 mg/kg/3 days). Further, our animals which were trained to self-administer cocaine (14 days, 0.25 mg/0.1 ml/infusion) did not show signs of post-synaptic adaptations. Therefore, species differences are unlikely to have contributed to this disparity. Furthermore, preliminary experiments in our laboratory indicate similar presynaptic effects in mice. A more likely explanation might be that in our study, all experimental procedures were carried out during the active (dark) phase while CPP procedures performed by Rao et al. (2013) occurred during the inactive (light) phase. Despite these differences in experimental procedures, both experiments indicate that cocaine induces synaptic plasticity in LH orexin circuitry (**Figures 1A, B**). These data, which indicate that addiction leads to an overstimulation of orexin neurons, may provide a mechanistic rationale for antagonizing the downstream actions of enhanced orexin signaling in addiction using SORAs and DORAs.

Furthermore, evidence exists that altered feeding behavior modifies LH orexin neuron circuitry at a cellular level. Specifically, work using an orexin-GFP mouse to selectively study the properties of orexin neurons showed an increase in miniature excitatory post-synaptic currents (mEPSCS), a measure of increased synaptic drive, in LH slices of mice that underwent mild food restriction for 24 h, as compared to normally-fed controls. Consistent with these electrophysiological findings, and similar to our LH addiction experiments, overnight food restriction promoted the formation of excitatory VGLUT2 synapses onto orexin neurons. These cellular effects of food restriction were rapidly reversed by re-feeding (Horvath and Gao, 2005), contrasting the addiction work where comparable cellular perturbations persisted beyond the removal of drug. Together, these behavioral and cellular studies highlight the potential impact for SORAs and DORAs on appetite and natural rewards.

Importantly, the source of the enhanced excitatory drive to orexin neurons remains to be determined. The LH receives significant glutamatergic inputs from other brain regions such as the prefrontal cortex, lateral septum and basolateral amygdala. For example, Morshedi and Meredith (2008) have shown that a sensitizing regimen of amphetamine upregulates Fos immunoreactivity in medial prefrontal cortex neurons projecting to the LH. Further, cocaine CPP has also been demonstrated to produce increased Fos immunoreactivity in the lateral septum, another area that projects to the LH (Sartor and Aston-Jones, 2012). It is also worth noting the possibility that local glutamatergic neurons may provide positive feedback to the orexin system in cocaine-exposed animals (Li et al., 2002; Jennings et al., 2013). Nonetheless, further studies are needed to confirm the likely source of these increased glutamatergic inputs onto orexin neurons in response to cocaine exposure and to determine how other classes of drugs might also rewire LH-orexin circuits. A final point that should be noted is that recent elegant work by Burdakov's group has demonstrated the existence of two subpopulations of orexin neurons in the LH (termed H-type and D-type), which can be distinguished electrophysiologically and anatomically (Schöne et al., 2011). It will be important for future studies to determine if drugs of abuse (or stress) differentially affect these orexin cell subtypes.

EFFECTS OF SORAs AND DORAs ON DRUG-SEEKING BEHAVIORS

A number of SORAs now exist, the most common being those that target the OxR1. These include SB-334867 (Porter et al., 2001; Smart et al., 2001), SB-674042 (Langmead et al., 2004), SB-408124 (Langmead et al., 2004), SB-410220 (Porter et al., 2001; Langmead et al., 2004), GSK1059865 (Gozzi et al., 2011) and, most recently, ACT-335827 (Steiner et al., 2013a) all of which have

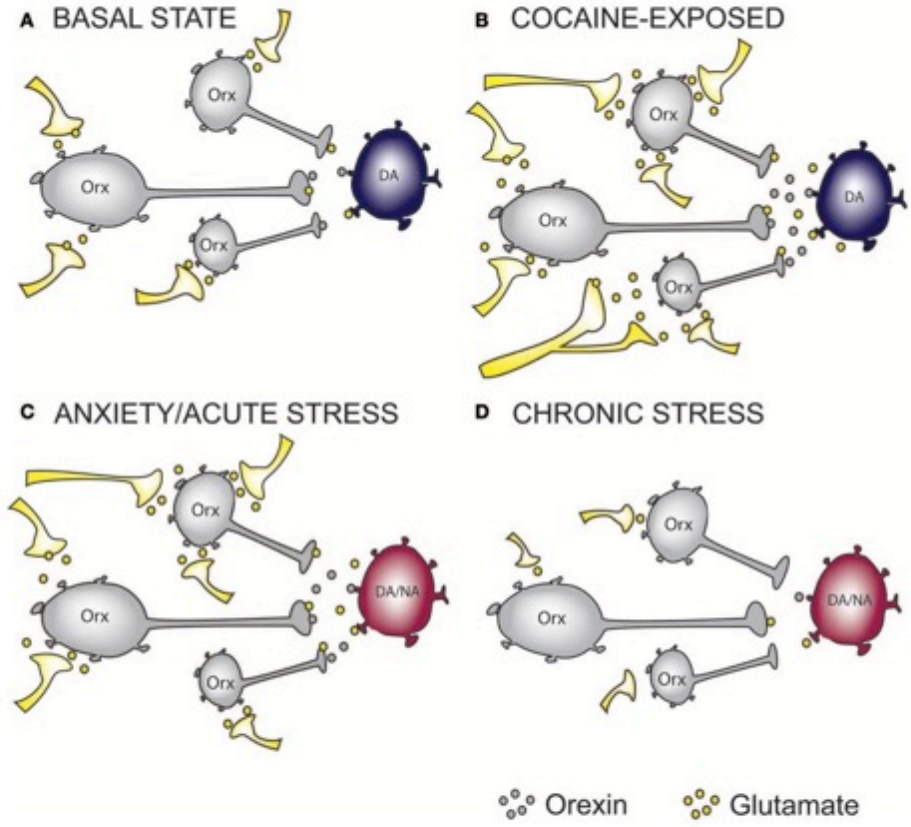

FIGURE 1 | The effect of cocaine and stress exposure on orexin neurons. **(A)** Under basal conditions, orexin (Orx) neurons in the lateral hypothalamus receive excitatory inputs, thus providing "normal" glutamatergic and orexinergic (excitatory) tone onto downstream projection targets such as ventral tegmental area (VTA) dopamine (DA) neurons. **(B)** Based on recent studies, we propose that chronic cocaine-exposure rewires glutamatergic inputs onto Orx neurons resulting in increased excitatory drive onto DA neurons in the VTA. **(C)** Anxiety and acute stress are able to cause enhanced excitatory drive to DA neurons in the VTA and Noradrenergic (NA) neurons in the Locus Coeruleus (LC), an important projection target of orexin neurons. **(D)** Glutamatergic inputs onto Orx neurons in chronically stressed animals are impaired resulting in reduced excitatory drive onto DA and/or NA neurons. On the basis of this simplified hypothetical mechanistic rationale, orexin receptor antagonism represents a possible therapeutic intervention to treat anxiety and addictions. In the case of reduced orexin system activity, as may occur in some forms of depression or in response to chronic stress, mechanisms to augment orexin function may be required. Whether chronic modulation of these systems can be achieved without precipitating the expression of an alternate neuropsychiatric state warrants careful consideration.

at least 50-fold selectivity for OxR1 over OxR2 (Scammell and Winrow, 2011; Zhou et al., 2011). Amongst these compounds, SB-334867 has been most widely studied in terms of behavioral pharmacology, largely due to the high selectivity, potency and availability of this drug (Scammell and Winrow, 2011; Zhou et al., 2011).

A large number of studies have demonstrated that OxR1 antagonists are effective at blocking addiction-related behaviors across a range of drug classes. With respect to self-administration behavior, treatment with SB-334867 effectively attenuates ethanol and nicotine self-administration under both low-effort fixed ratio (FR) and higher-effort progressive ratio (PR) schedules (Lawrence et al., 2006; Schneider et al., 2007; Hollander et al., 2008; LeSage et al., 2010; Jupp et al., 2011; Martin-Fardon and Weiss, 2012). Interestingly, in the case of cocaine, SB-334867 has no effect on self-administration behavior under FR1 or FR3 conditions (Smith et al., 2009; Espana et al., 2010) but does attenuate self-administration under higher-effort schedules, including FR5 (Hollander et al., 2012) and PR (Borgland et al., 2009; Espana et al., 2010). These findings imply that in general, the orexin system has limited actions on the primary rewarding effects of cocaine, but is important to overcome increased motivational demands or effort to procure rewards. In contrast, SB-334867 effectively blocks the expression of CPP for amphetamine (Hutcheson et al., 2011) and morphine (Sharf et al., 2010), a widely used measure of the rewarding effects of drugs of abuse. Systemic SB-334867 treatment also attenuates reinstatement of cocaine-seeking behavior elicited by drug cues (Smith et al., 2009), contexts (Smith et al., 2010) and footshock stress (Boutrel et al., 2005), but not a cocaine prime (Mahler et al., 2013). Similarly, SB-334867 attenuates cue- and stress-induced reinstatement of alcohol seeking (Lawrence et al., 2006;

Richards et al., 2008; Jupp et al., 2011) as well as cue-induced, but not primed, heroin seeking (Smith and Aston-Jones, 2012). SB-334867 also attenuates cue- (Plaza-Zabala et al., 2013), but not footshock- (Plaza-Zabala et al., 2010) induced reinstatement of nicotine seeking—the latter result being a particularly surprising outcome. Thus, a wealth of preclinical studies support the premise that the use of OxR1 SORAs have the potential to decrease addiction-related behaviors, suggesting they may have clinical value in preventing relapse in abstinent patients.

In contrast to OxR1 antagonists, fewer studies have assessed the effects of selective OxR2 antagonists on addiction-related behaviors. This is likely due to the more recent development of these compounds and a predicted increase in the likelihood of sedation (Zhou et al., 2011). Selective OxR2 antagonists include JNJ-10397049 (McAtee et al., 2004; Dugovic et al., 2009), EMPA (Malherbe et al., 2009a), and TCS-OX2-29 (Hirose et al., 2003), all of which have at least a 250-fold selectivity for OxR2 (Scammell and Winrow, 2011; Zhou et al., 2011). Studies exploring the effects of these OxR2 SORAs have reported less consistent effects on drug-motivated behaviors compared to the OxR1 SORA SB-334867. For example, Shoblock et al. (2010) showed that JNJ-10397049 dose-dependently deceased ethanol self-administration, as well as the acquisition, expression and reinstatement of ethanol CPP. In contrast, Brown et al. (2013) showed that TCS-OX2-29 attenuated ethanol self-administration but had no effect on cue-induced reinstatement of extinguished ethanol seeking. Interestingly, these authors identified the nucleus accumbens core (NACc) as an important site for OxR2 signaling, as infusions of TCS-OX2-29 into the NACc, but not shell, reduced ethanol self-administration. In contrast, OxR2 SORAs have been shown to have no effect on cocaine self-administration or reinstatement (Smith et al., 2009) or the expression of nicotine withdrawal symptoms (Plaza-Zabala et al., 2012). Thus, regarding SORAs, the case for OxR1-based therapies in treating addiction is better developed than for antagonists targeting OxR2.

With respect to DORAs, a large number of these compounds have been developed, prompted largely by their potential as a novel treatment for insomnia. Indeed, at least three pharmaceutical companies (GSK, MERCK, Actelion) have initiated clinical trials investigating the utility of DORAs in modulating the sleep-wake cycle. These DORAs include almorexant (ACT-078573; Brisbare-Roch et al., 2007; Malherbe et al., 2009b); suvorexant (MK-4305; Cox et al., 2010), Merck DORA-1 (Bergman et al., 2008), Merck DORA-5 (Whitman et al., 2009), and SB-649868 (Renzulli et al., 2011). Only a limited number of studies have examined the effects of DORAs on addiction-related behaviors, with this work focusing almost exclusively on almorexant. For example, systemic and intra-VTA almorexant treatment was shown to attenuate ethanol self-administration (Srinivasan et al., 2012). Similarly, systemic almorexant blocked nicotine self-administration behavior (LeSage et al., 2010). Interestingly, whilst almorexant attenuated the expression of CPP to high doses of cocaine and amphetamine, it had no effect on morphine CPP (Steiner et al., 2013c). This study also showed that almorexant reduced the expression of behavioral sensitization to morphine but not to cocaine or amphetamine (Steiner et al., 2013c).

EFFECT OF SORAs AND DORAs ON FOOD AND NATURAL REWARD-SEEKING BEHAVIOR

An important consideration with respect to the potential clinical application of orexin receptor antagonists is the effect of these compounds on other appetitive behaviors, including food seeking. Indeed, studies carried out immediately following the discovery of the orexin peptides firmly implicated orexin in feeding behavior, with intracerebroventricular (i.c.v.) infusions of orexin shown to increase food consumption (Sakurai et al., 1998) whereas systemic treatment with SB-334867 was found to block feeding behavior (Haynes et al., 2000).

Since these initial demonstrations, subsequent studies have sought to investigate whether doses of orexin receptor antagonists that are required to block drug seeking also affect feeding behavior. For example, Martin-Fardon and colleagues recently showed that systemic administration of SB-334867 (1–10 mg/kg, i.p) attenuated reinstatement of cocaine seeking, but not sweetened-condensed milk seeking, elicited by discriminative cues (Martin-Fardon and Weiss, 2014). Similarly, Jupp et al. (2011) showed that systemic injections of SB-334867 (5 mg/kg) were sufficient to reduce responding for both ethanol and sucrose under an FR3 schedule of reinforcement. However, SB-334867 (5 mg/kg) attenuated responding for ethanol but not sucrose under a PR schedule. The authors suggested that the contribution of orexin A to motivation for alcohol is independent of non-specific effects on appetitive drive. Likewise, Hollander et al. (2012) showed that systemic injections of SB-334867 (2–4 mg/kg) reduced cocaine self-administration, but had no effect on responding for food rewards under an FR5 schedule. Comparable effects have also been shown with doses of SB-334867 that attenuate responding for nicotine (LeSage et al., 2010). Further, we have previously reported that intra-VTA infusions of SB-334867, at doses that suppress cue-induced cocaine seeking, had no effect on reinstatement for a natural reward (sweetened condensed milk; James et al., 2012). With respect to OxR2 antagonists, Brown et al. (2013) showed that central infusions of 100 μg TCS-OX2-29 reduced self-administration of ethanol, but had no effect on sucrose self-administration. In contrast however, LeSage et al. (2010) showed that systemic treatment with the DORA almorexant attenuated responding for both food pellets and nicotine. Similarly, systemic almorexant suppressed ethanol self-administration, and responding for sucrose (Srinivasan et al., 2012).

Taken together, these data indicate that a putative therapeutic window exists in which SORAs could be used to treat addiction-relevant behaviors, including 'relapse,' without producing 'off-target' effects on natural reward-seeking behavior. Conversely, it appears likely that the use of DORAs in the treatment of addiction may be associated with a risk of interfering with natural appetitive processes or promoting sedation. It is also important to acknowledge that there are very few studies that have assessed the effect of subchronic or chronic orexin receptor antagonism on drug or food-motivated behavior (discussed below).

RECENT PROGRESS IMPLICATING THE OREXIN SYSTEM IN STRESS-RELATED NEUROENDOCRINE RESPONSES

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is an important component of the adaptive response to stress (Dayas

et al., 2001; Day and Walker, 2007; Ulrich-Lai and Herman, 2009). In this regard it is noteworthy that the neuroendocrine paraventricular nucleus (PVN), the apex of the HPA axis, contains both OxR1 and OxR2 and that both receptor sub-types are expressed in the anterior and intermediate lobe of the pituitary gland (Trivedi et al., 1998; Date et al., 2000). Consistent with this anatomical evidence, several reports have suggested that orexins can modulate the HPA axis. For example, i.c.v. injections of orexin have been shown to increase Fos-protein expression in PVN corticotropin-releasing factor (CRF) neurons (Sakamoto et al., 2004), provoke adrenocorticotropin-releasing hormone (ACTH) release from the anterior pituitary, and increase the release of corticosterone from the adrenal glands (Jászberényi et al., 2000; Kuru et al., 2000; Russell et al., 2001; Moreno et al., 2005).

Importantly, researchers using systemic administration of orexin receptor antagonists have reported less consistent effects on HPA axis activity as would have been predicted from studies assessing HPA axis activity after orexin peptide infusions. For example, systemic injections of the OxR1 antagonist GSK-1059865 did not alter corticosterone responses to the pharmacological stressor yohimbine in rats (Gozzi et al., 2011, 2013). Further, oral treatment with almorexant had no effect on basal, social interaction, novelty or restraint stress-induced corticosterone release (Steiner et al., 2013b). Systemic SB-334867 administration also did not attenuate withdrawal-induced increases in plasma corticosterone release (Laorden et al., 2012) despite reducing the physical symptoms associated with morphine withdrawal in Wistar rats.

These equivocal effects of orexin on HPA axis function are somewhat surprising given the abundance of orexin receptors in the PVN and pituitary (Trivedi et al., 1998). Interestingly, i.c.v. administration of TCS-OX2-29, a selective OxR2 antagonist, attenuated swim stress-induced increases in plasma ACTH release (Chang et al., 2007) and, intra-PVT infusions of SB-334867 attenuated the ACTH response to restraint but only following repeated swim stress (Heydendael et al., 2011). Thus, any role of orexin in HPA axis control might depend on prior stress exposure, the category of stressor e.g., physical vs. psychological, its intensity and duration, or whether in repeated stress experiments, homotypic or heterotypic stressors are applied.

Despite the above data, exposure to psychological and physical stressors increase surrogate indices of orexin system function. For example, increased Fos-protein expression is observed in orexin neurons following exposure to acute footshock (Harris and Aston-Jones, 2006), fear-associated contexts and novel environments (Furlong et al., 2009). Interestingly, similar effects are not observed following acute immobilization stress (Furlong et al., 2009), however this form of stress does increase orexin mRNA levels in the LH (Ida et al., 2000). An explanation for these contrasting findings remains to be determined, however, one interpretation is that only sufficiently intense or salient stimuli recruit the orexin system and that prior arousal state strongly influences the likelihood of orexin system recruitment. With respect to pharmacological stressors, increased Fos-expression is observed in orexin neurons following systemic injections of the anxiogenic drug FG-7142 (Johnson et al., 2012), caffeine (Johnson et al., 2012) and intravenous administration of sodium lactate (Johnson et al., 2010). Additionally, systemic administration of OxR1 antagonist GSK-1059865 has demonstrated functional inhibition in stress-relevant brain regions such as the NAC, dorsal thalamus, amygdala, and ventral hippocampus following the administration of yohimbine (Gozzi et al., 2013).

Acute stress also appears to have long lasting effects on orexin gene expression. For example, 2 weeks following a single session of footshock stress, increased prepro-orexin mRNA levels were observed in both the medial and lateral divisions of the hypothalamus (Chen et al., 2013). Elevated levels of orexin-A peptide in the cerebrospinal fluid (CSF) of Wistar rats have also been demonstrated following a short-term forced swimming paradigm (Martins et al., 2004). In addition, increased orexin mRNA was observed in rats immediately following morphine withdrawal (Zhou et al., 2006). Consistent with this Fos-activity mapping of orexin cell reactivity to stress, acute orexin peptide infusions evoke anxiety-like behavior. Specifically, i.c.v. administration of orexin-A produced anxiogenic-like effects i.e., increased the time spent in the closed arms of the elevated plus maze and the time spent in the dark compartment of the light-dark test (Suzuki et al., 2005). Orexins have also been shown to modulate the activity of extrahypothalamic CRF systems with i.c.v. injections of orexin-A found to increase the percentage of CRF cells that express Fos in the central amygdaloid nucleus (Sakamoto et al., 2004). Together, this evidence forms the basis for the hypothesis that manipulation of the orexin system using SORAs and DORAs will likely also impact on stress–induced anxiety-like behavior.

EFFECT OF SORAs AND DORAs ON ACUTE BEHAVIORAL STRESS REACTIVITY AND ANXIETY-LIKE BEHAVIOR

Several recent preclinical studies indicate that SORAs and DORAs have a limited effect on basal/non-stress evoked behavioral responses including anxiety-like behavior. For example, systemic treatment with SB-334867 in both rats and mice had no effect on activity in the elevated plus maze and social interaction task, two common tests of anxiety-like behavior (Johnson et al., 2010; Rodgers et al., 2013). However, intra-PVT SB-334867 infusions resulted in decreased anxiety-like behavior in the elevated plus maze (Heydendael et al., 2011). This could indicate an important role for orexin signaling in the PVT in regulating basal arousal or anxiety state.

Importantly, SORAs reliably attenuated anxiety-like behavior evoked by an acute psychological or physical stressor. For example, systemic SB-334867 administration reduced the expression of anxiety-like behavior induced by acute nicotine (Plaza-Zabala et al., 2010) and sodium lactate injections (Johnson et al., 2010) as well as cat odor (Staples and Cornish, 2014). Administration of SORAs also reduced physiological responses such as increased heart rate and body temperature produced by exposure to acute i.p injection stress (Rusyniak et al., 2012). With regards to OxR2 antagonists, intra-PVT infusions of TCS-OX2-29 reduce anxiety-like behavior elicited by footshock stress (Li et al., 2010) suggesting a role for orexin signaling through the OxR2 in stress-relevant behaviors.

There are also several recent animal studies that have explored the role for the orexin system in anxiety-like behavioral responses to conditioned cues that predict the presentation of a fearful

stimulus, such as footshock. This approach has been used to explore brain mechanisms responsible for generating panic disorder and post-traumatic stress disorder in humans (Grillon, 2008). In male Sprague-Dawley rats, Sears et al. (2013) administered SB-334867 i.c.v. before fear conditioning and demonstrated an impairment in conditioned stimulus (CS)-induced freezing behavior 24 h after CS/unconditioned stimulus (US) presentation. Further, systemic injections of TCS-1102 decreased conditioned and generalized fear and anxiety-like behavior in an open field in rats previously exposed to footshock stress (Chen et al., 2013). Moreover, OxR1 knockout mice demonstrated a reduction in freezing behavior in both cued and contextual fear conditioning. Interestingly, OxR2 knockout mice also showed reduced freezing behavior to contextual but not conditioned fear (Soya et al., 2013). Consistent with a role for both receptor subtypes in aspects of conditioned fear responses, Steiner et al. (2012) reported that oral administration of almorexant reduced fear-potentiated startle in response to a CS but found no change in elevated plus maze behavior. Interestingly, human genetic studies have linked a polymorphism in Orx-2 receptor gene with panic disorder (Annerbrink et al., 2011).

Together these data indicate that exposure to acute stress and the expression of anxiety-like behavior is generally associated with increases in orexin system activity. Further, orexin antagonists appear to be potential candidates for the suppression of anxiety-like behavior and maladaptive stress-reactivity. Importantly, as is outlined below, recent evidence suggests that chronic suppression of the orexin system may be associated with the development of depression-like symptomology. These findings have significant implications for the potential use of orexin antagonists in the treatment of neuropsychiatric disorders.

EFFECT OF CHRONIC STRESS ON THE OREXIN SYSTEM AND IMPLICATIONS FOR DEPRESSION

In recent human studies, a polymorphism in Orx-1 receptor gene has been associated with major mood disorders and increased orexin peptide levels were correlated with positive emotions and social interaction (Rainero et al., 2011; Blouin et al., 2013). Non-genetic factors, such as chronic stress, have also implicated orexin in the etiology of depression (Katz et al., 1981; Kendler et al., 1999; Charney and Manji, 2004; Russo and Nestler, 2013). This link has been studied in animal models typically involving extended periods of psychological stress exposure (Lutter et al., 2008). Increasing evidence from these types of studies links chronic stress exposure to a downregulation of orexin system activity. For example, mice exposed to the well characterized social defeat model of chronic stress, which evokes symptoms thought to mimic depression in humans, display reduced orexin mRNA expression, lowered orexin cell number, and diminished levels of orexin A and orexin B peptide (Lutter et al., 2008; Nocjar et al., 2012). Similarly, Wistar-Kyoto (WKY) rats, which exhibit a depressive-like behavioral phenotype, express lower numbers of orexin neurons and a smaller orexin soma size compared to Wistar rats (Allard et al., 2004). The WKY rat strain also exhibits reduced prepro-orexin mRNA levels and orexin A immunoreactivity is reduced in the hypothalamus, thalamus, septum and amygdala (Taheri et al., 2001).

Early life stress (ELS) is a known risk factor for the development of stress-related mood disorders in adulthood (Graham et al., 1999). Surprisingly though, work has shown that ELS in fact increases frontal cortical OxR1 and hypothalamic orexin A levels in adulthood (Feng et al., 2007). Given these results, we recently investigated the effects of ELS on the reactivity of the orexin system to a second psychological stressor in adulthood. Both male and female rats exposed to neonatal maternal separation displayed a hypo-active orexin cell response to stress in adulthood. These animals also displayed reduced open field behavior but notably no overt anxiety-like behavior was observed on an elevated plus maze. Our interpretation of these data is that the ELS procedures evoked behaviors more akin to a depression-like profile after stress however further behavioral tests will be necessary to further explore this hypothesis (Campbell et al., 2013).

What effect a second stressor, such as restraint in adulthood, might have on LH-orexin circuits is unknown. This would seem important given the behavioral effects of this "two-hit" paradigm outlined above (Campbell et al., 2013). It is possible that increased drive to this system reflects an attempt to enhance orexin activity and prevent the expression of depression-like behavior. It may be that in response to a significant subsequent stressor, the reduced functional integrity at a synaptic and molecular level is unmasked, which may then develop into a depressive-like state. Regardless, taken together with the Fos data outlined above, our findings suggest that ELS can have long-term impacts on the normal functioning of orexin cells.

Consistent with a process where ELS can induce orexin cell dysfunction and depression-like behaviors, clinical evidence supports the possibility that decreased orexin signaling might promote depression-like behaviors. For example, reduced concentrations of orexin A were reported from CSF samples of adults with major depressive disorder and chronic, combat-related post-traumatic stress disorder (Brundin et al., 2007a,b, 2009; Strawn et al., 2010). Additionally, reduced orexin A mRNA has been correlated with increased scores on the Hamilton rating scale for depression (Rotter et al., 2011). Interestingly, infusions of orexin peptides into the ventricles of rats have been shown to have antidepressant-like effects. For example, i.c.v. administration of orexin reduces the duration of immobility in the forced swim test, and this effect is blocked by the administration of the OxR1 antagonist, SB-334867 (Ito et al., 2008). Thus, it is possible that in some forms of depression, increasing orexin system signaling may have therapeutic benefit. In line with this interpretation, we recently found that a period of exercise in adolescence prevented reductions in orexin system function and the expression of stress-related behavior in rats exposed to ELS (Campbell et al., 2013).

Despite the above work, it is important to acknowledge that there are both preclinical animal and human data that do not support a link between a hypoactive orexin system and depression. For example, Mikrouli et al. (2011) demonstrated that the Flinders Sensitive Line, considered a genetic rat model of depression, displayed elevated, not depressed, levels of orexin neurons compared to controls. Further, Nollet et al. (2011) demonstrated that mice subjected to unpredictable chronic mild stress displayed increased depressive-like behavior following the tail suspension

test but presented with elevated orexin neuron activity in the DMH and PFA subregions of the hypothalamus. Interestingly, these authors were able to reverse this elevation in orexin cell activity with 6 weeks of fluoxetine treatment. Similarly, exposure to unpredictable chronic mild stress produced depressive-like behaviors in the tail suspension test, elevated plus maze and resident-intruder task; and 7 weeks exposure to the DORA almorexant produced an antidepressant-like behavioral effect in these tasks (Nollet et al., 2012). And, in a recent study, OxR1 mRNA expression in the amygdala was reported to be positively correlated with increased depressive-like behavior in the forced swim test (Arendt et al., 2013) - however it is possible that this effect might be caused by downregulated orexin system function. Finally, a recent study reported that decreased depressive-like behavior is observed in OxR1 knockout mice, whereas OxR2 knockout mice exhibit *increased* depressive-related behavior, possibly pointing to a differential role for OxR1 vs. OxR2 in the regulation of these behaviors (Scott et al., 2011). These authors highlight the fact that behavioral pharmacology studies typically use the non-selective OxR agonist orexin-A, along with SORAs at doses that are potentially non-selective *in vivo*, therefore making it difficult to differentiate roles for OxR1 vs. OxR2 in the regulation of depression-like behavior.

In human studies, Salomon et al. (2003) reported that orexin A CSF levels were higher in depressed patients and treatment with sertraline, an antidepressant drug, resulted in an attenuation of CSF orexin levels. Furthermore, a positive correlation between orexin plasma concentrations, depressive symptoms and global distress indices on the brief symptom inventory is seen following alcohol withdrawal (von der Goltz et al., 2011). Finally, Schmidt et al. (2011) failed to find any association between CSF orexin A levels and depression.

Taken together, these results suggest that acute stress may activate the orexin system in order to enhance an animal's ability to cope or adapt appropriately to a potential threat (**Figure 1C**). If these stressors persist, chronic or repeated exposure to stress may downregulate orexin system function (**Figure 1D**). Hypoactivity of the orexin system may impair an animal's ability to adapt to stress and lead to the expression of depressive-like behavior. Data from human and animal studies not supporting this link may reflect the heterogeneous nature of depression—i.e., depression presenting with and without anxiety or anxiety presenting with or without depression. Supporting this conclusion, Johnson et al. (2010) found that patients exhibiting panic anxiety displayed increased CSF orexin levels compared to patients exhibiting panic anxiety with comorbid major depressive disorder. It will be important for human and animal studies exploring the link between the orexin system dysfunction and neuropsychiatric conditions to consider the heterogeneous nature of these conditions.

POTENTIAL PITFALLS FOR APPROACHES TO TREAT NEUROPSYCHIATRIC DISORDERS USING OREXIN RECEPTOR ANTAGONISTS

As outlined above, significant progress has been made in our understanding of the contribution of the orexin system to normal and "pathological" behavior. In the case of addiction, there seems sufficient evidence to conclude that the orexin system is important for drug-seeking behavior, particularly for relapse-like behavior provoked by drug-cues and stress but not by drug itself. Further, several studies indicate that orexin antagonists, and in particular selective OxR1 antagonists, can reduce drug-seeking at doses that have minimal effects on natural reward-seeking behavior (Jupp et al., 2011; Hollander et al., 2012; James et al., 2012; Brown et al., 2013). Comparison of orexin's role in drug taking vs. seeking behavior also highlights that orexin receptor antagonists have effects on rewarded self-administration, but importantly these effects generally emerge only under high effort schedules of reinforcement. These data combine to produce a compelling case that orexin receptors represent promising targets for the treatment of addiction. This is particularly true given the long-established interaction between addiction and maladaptation of natural reward seeking brain pathways. For example, increased excitatory drive to the orexin system is thought to heighten orexin signaling in key reward-seeking regions such as the VTA and may contribute to the persistent plasticity within dopamine neurons seen after long-term cocaine self-administration and increased relapse vulnerability (Chen et al., 2008; James et al., 2012)

It is important given the clinical promise of SORAs and DORAs for neuropsychiatric disorders that studies continue to assess the potential for off-target effects, tolerance to prolonged orexin receptor blockade and differential or counter-regulatory effects on OxR1 vs. 2, as well as any possible compensatory adaptations that may occur in other hypothalamic neuropeptide systems in response to chronic orexin receptor blockade. Studies to date indicate a limited profile of chronic SORA and DORA tolerance. For example, Steiner et al. (2013c) showed that 12 days of chronic almorexant treatment had no effect on the maintenance of CPP or locomotor sensitization in animals exposed to cocaine, morphine or amphetamine. However, as mentioned above, Nollet et al. (2012) exposed mice to chronic almorexant treatment (7 weeks), which produced an antidepressant-like effect in the tail suspension test, elevated plus maze and resident-intruder task following exposure to unpredictable chronic mild stress. Interestingly, chronic treatment with the OxR1 antagonist ACT-335827 (4 weeks) did not alter total energy intake in cafeteria diet fed rats compared to controls (Steiner et al., 2013b). In contrast, chronic SB-334867 treatment (14 days) had anti-obesity effects in a model of genetically obese mice by reducing food intake and body weight gain over the 14 day period (Haynes et al., 2002).

In one of the few examples where repeated doses of an orexin antagonist have been studied for addiction and relapse prevention, Zhou and colleagues demonstrated that chronic SB-334867 exposure resulted in a complex pattern of effects (Zhou et al., 2011). Specifically, they found that repeated SB-334867 exposure prior to extinction sessions resulted in reduced cocaine-seeking behavior in rats during extinction; however, repeated SB-334867 treatment during extinction increased cue-induced reinstatement and had no effect on cocaine-primed reinstatement. Importantly, McNally and colleagues have also shown that cocaine and alcohol-seeking do not necessarily evoke a specific drug context-related activation of the orexin system, rather,

recruitment of this pathway is necessary but not sufficient for drug-seeking behavior (Hamlin et al., 2007, 2008). It is also interesting to note that the OxR1 antagonist, SB-334867, has been shown to reduce cue-induced reinstatement of cocaine seeking behavior in male rodents yet no effect was seen in female rats (Zhou et al., 2012). Future work should focus on differentiating arousal vs. reward related function of the orexin neurons in the context of addiction. Sex-specific effects of stress and drug exposure on orexin circuitry also warrant further scrutiny.

With respect to the potential use of orexin receptor antagonists for anxiety and depression, recently, Johnson et al. (2010, 2012) proposed the use of OxR1 antagonists in the treatment of panic disorder. Certainly, the available data appears to support an important role for orexin signaling in ameliorating anxiety-like states in animal models. Studies employing chronic stress paradigms, however, suggests that a more complicated picture exists and that the effects of repeated SORA or DORA treatment may be unpredictable (Zhou et al., 2012). Emerging data indicates that ELS and chronic stress can downregulate the activity of the orexin system in response to chronic stress (Lutter et al., 2008; Nocjar et al., 2012; Campbell et al., 2013). There is also a developing clinical literature indicating that depression may be associated with decreased orexin system function (Brundin et al., 2007a,b, 2009). These studies raise potential concerns for the long-term use of orexin antagonists in the treatment of addiction and anxiety disorders, as long-term suppression of the orexin system may precipitate depressive-like symptoms. Similarly, the emergence of anxiety or depression under conditions of augmented orexin system function will need to be carefully considered. Therefore, a greater understanding of the changes to orexin receptor expression in relevant brain regions will be necessary to confidently predict the potential outcomes of therapeutic manipulation of orexin signaling under these conditions. Further we propose that a thorough evaluation of chronic and subchronic orexin receptor antagonism in preclinical animal models of anxiety and depression is necessary. Together, such approaches may be able to identify therapeutic dosing regimens with preferential effects on the different aspects for the orexin system that influence mood, as well as drug- and natural-reward behaviors. Finally, the evidence that drugs of abuse or stress can rewire inputs onto orexin neurons indicates that targeting these changes within the LH might offer an alternative strategy. This would negate the mixed downstream effects of SORAs and DORAs in mood, addiction and reward by reducing aberrant orexin cell activity at the site of dysfunction, rather than simply masking the downstream effects using antagonists. For example, our group is currently investigating the changes in LH-orexin circuits responsible for the increased excitatory drive observed after cocaine or ELS.

In conclusion, significant progress has been made toward an understanding of the role of the orexin system in normal and pathological behaviors. Unsurprisingly, given the widespread projections of the orexin system, the role for these neurons crosses many domains including basic physiological responses to more complex functions. Our view is that only a comprehensive dissection of the changes in LH-circuit function, both within the hypothalamus and in target regions of these neurons, will reveal appropriate therapeutic avenues to augment or suppress dysregulated orexin function in neuropsychiatric and neurological disease states.

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CONCLUSIONS AND AIMS

The lateral hypothalamus (LH), in particular the lateral hypothalamic orexin system, plays an important role in normal behaviour including reward-seeking and reactivity to stress. Additionally, both acute and chronic stress might alter normal functioning of this system leading to neuropsychiatric disorders. Given that early life stress (ELS) is an antecedent for the development of neuropsychiatric disease, *Chapter 1* of my thesis used behavioural and immunohistochemical techniques to examine whether early life maternal separation altered orexin system function and whether this lead to a stress-induced behavioural phenotype in adulthood. I found that ELS resulted in a reduced number of Fos-positive orexin neurons in both male and female rats. This ELS also reduced exploratory behaviour in the open field task without affecting anxiety-like behaviour in the elevated plus maze. Interestingly, adolescent exercise reversed both behavioural and orexin deficits in male but not female rats. These findings highlighted the plasticity of the orexin system in response to ELS however the behavioural phenotype exhibited by these maternally separated rats remains unclear.

Following the completion of this Chapter, I was awarded a travel grant to go to the National Institute on Drug Abuse (NIDA), USA, and learn novel chemogenetic techniques to manipulate the neural circuitries involved in addiction and apply these techniques to our research here at Newcastle. During this period (8 months) I completed *Chapter 2* of my thesis, which examined the negative consequences of excessive alcohol use in rats. I used behavioural approaches in which alcohol preferring ‘P’ rats were trained to self-administer alcohol in one context (Context A), then alcohol-reinforced lever presses were punished using foot shock in a different context (Context B) and finally rats were tested for relapse to alcohol seeking in contexts A and B. I also used retrograde tracer techniques combined with Fos immunohistochemistry to demonstrate that context-induced relapse after punishment is associated with selective activation of the ventral subiculum to nucleus accumbens shell projection. Finally, I used a dual-virus approach to restrict expression of the inhibitory kappa opioid-receptor based DREADD

(KORD) in ventral subiculum to nucleus accumbens shell projection neurons. Selective inhibition of this pathway decreased context-induced relapse to alcohol seeking.

Upon returning to Newcastle, I sought to continue on with my maternal separation experiments and incorporate the novel designer receptors exclusively activated by designer drugs (DREADD) technique into my work. The behavioural phenotype exhibited by my maternally separated animals from Chapter 1 is unlikely to be anxiety given the null effect of maternal separation on elevated plus maze behaviour. Based on this, *Chapter 3* of my thesis aimed to examine the behavioural phenotype in adulthood produced by maternal separation and functionally implicate the lateral hypothalamus using DREADD technology. Maternally separated male rats underwent forced swim behavioural testing to examine behavioural despair in adulthood, a cardinal symptom of depression. These rats also underwent sucrose self-administration using a progressive ratio schedule of reinforcement to examine the motivation for natural reward. Behavioural results indicated a reduction in lever responding for sucrose in maternally separated rats compared to controls. No differences were found across neonatal treatment in forced swim behaviour. Given the known involvement of the lateral hypothalamus in reward behaviour, I next used an excitatory designer receptor (hM3D-Gq) to activate cells in the lateral hypothalamus and examine the effect on the motivation for sucrose reward. Activating the lateral hypothalamus was able to increase lever responding for sucrose in maternally separated animals back up to control level. Following this, I examined the hypothalamus for Fos-protein and orexin immunohistochemistry.

Maternal separation is a psychological early life stressor known to increase the vulnerability to neuropsychiatric disease in later life. Interestingly, other forms of early life stress, such as physiological stress, also contributes to the onset of psychopathology in adulthood. *Chapter 4* of my thesis sought to examine the effects of an early life immune challenge on the orexin system. Male rats were exposed to lipopolysaccharide (LPS) during the neonatal period and formalin-induced inflammatory pain in adulthood. Using Fos-

protein and orexin immunohistochemistry and behavioural approaches, I found that rats treated with LPS during the neonatal period had altered licking and grooming responses to inflammatory pain in adulthood. Interestingly, this was associated with an increase in the number of Fos-positive orexin neurons.

THESIS CHAPTERS

CHAPTER ONE

Exercise reverses the effects of early life stress on orexin cell reactivity in male but not female rats.

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Erin J. Campbell	Designed and performed research, analyzed data and wrote the manuscript	
Morgan H. James	Designed and performed research, analyzed data and wrote the manuscript	
Frederick R. Walker	Designed the research and wrote the manuscript	
Doug W. Smith	Designed the research and wrote the manuscript	
Heather N. Richardson	Wrote the manuscript	
Deborah M. Hodgson	Designed the research and wrote the manuscript	

Christopher V. Dayas	Designed research, analyzed data and wrote the manuscript	

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Exercise reverses the effects of early life stress on orexin cell reactivity in male but not female rats

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Early life stress (ELS) is a known antecedent for the development of mood disorders such as depression. Orexin neurons drive arousal and motivated behaviors in response to stress. We tested the hypothesis that ELS alters orexin system function and leads to an altered stress-induced behavioral phenotype in adulthood. We also investigated if voluntary exercise during adolescent development could reverse the ELS-induced changes. Male and female Wistar rats were subjected to maternal separation stress on postnatal days (PND) 2-14. A subset of animals was given access to running wheels in late adolescence (1 hr/day, PND40-70). In adulthood, rats were exposed to restraint stress and then tested on the open field (OF) and elevated plus maze (EPM). Brains were processed for Fos-protein and orexin or tyrosine hydroxylase immunohistochemistry. Restraint stress stimulated Fos-protein expression in perifornical area orexin cells, the paraventricular hypothalamic nucleus, and paraventricular thalamic nuclei, but this neuronal response was dampened in male and female rats exposed to ELS. ELS also reduced exploration in the OF, without affecting EPM behavior. These neural and behavioral changes are consistent with a depressive-like phenotype. Adolescent exercise reversed the orexin and behavioral deficits in ELS males. Exercise was not protective in females, although this may be due to sex differences in running behavior. Our findings highlight the inherent plasticity of the orexin system—a trait that may lead to a state of pathological rewiring but could also be treated using non-pharmacological approaches. We also highlight a need to better understand the sex-specific changes in orexin circuits and stress-related pathology.

Keywords: orexin, hypocretin, stress, maternal separation, sex-differences, exercise, plasticity, hypothalamus

INTRODUCTION

Early life stress (ELS) is a major risk factor for the emergence of mood-related disorders such as depression and anxiety in adulthood (Danese et al., 2008). Preclinical studies show that separation of rat pups from their mother during the neonatal period (known as maternal separation) also increases vulnerability to anxiety- and depression-like behavior in adulthood (Winslow and Insel, 1991). The impact of ELS on the brain is dramatic and includes maladaptations to the neuroendocrine hypothalamus (i.e., the paraventricular nucleus; PVN) and associated feedback circuits (Meaney et al., 1996, 2007). Importantly, other hypothalamic systems are known to influence autonomic, neuroendocrine, and behavioral responses to stress, but there have been few studies addressing the impact of ELS on these non-neuroendocrine cell groups. For example, cell groups within the lateral hypothalamus (LH) have the capacity to influence a number of stress-relevant behavioral adaptations, including changes in arousal and reward status (Harris and Aston-Jones, 2006; Furlong et al., 2009). Dysregulation of these LH systems by ELS could significantly increase the risk for development of anxiety and depression in later life.

Of particular interest in this context are the orexin (hypocretin) neurons that are now known to be central to LH-mediated changes in arousal and motivational states (Harris and Aston-Jones, 2006; James et al., 2011, 2012; Johnson et al., 2012). Acute stress robustly increases activation of orexin neurons (Ida et al., 2000; Furlong et al., 2009), whereas chronic stress appears to have an opposite effect (Lutter et al., 2008; Nocjar et al., 2012). The ability of chronic stress to restrict orexin activity is particularly interesting, as evidence has recently emerged linking low orexin system function with depressive symptoms in humans (Brundin et al., 2007, 2009). Surprisingly, the effect of ELS on orexin neuron function in adulthood has not been directly tested. Therefore, the primary aim of this study was to investigate the effects of ELS on orexin system function following psychological stress exposure in adulthood.

Non-pharmacological approaches to produce or augment antidepressant/anxiolytic action have significant clinical relevance and appeal. Both clinical and preclinical studies suggest that physical activity or exercise can produce antidepressant-like effects (Greenwood et al., 2003; Lapmanee et al., 2013). At present however, it is unclear whether the antidepressant or anxiolytic

effects of physical activity might be linked to improvement in LH-orexin system function. Thus, a secondary aim of the present study was to investigate the possible preventative effects of physical activity on ELS-induced maladaptive orexin cell responses to stress in adulthood. Finally, because very few studies have examined the sex-specific effects of exercise on stress-related behavior, we carried out our experiments in both male and female rats.

METHODS AND MATERIALS

ETHICS STATEMENT

All procedures performed were approved by the University of Newcastle Animal Care and Ethics Committee, and were carried out in accordance with the New South Wales Animal Research Act.

ANIMALS

Ten experimentally naïve Wistar dams were obtained from the University of Newcastle Animal house and bred with two experimentally naïve males in the University of Newcastle vivarium. A total of 34 male and 39 female offspring were included in the study. As per previous studies (Caldji et al., 2000; Weaver et al., 2007; Nakamura et al., 2011), litters were not standardized to a fixed number of pups or male/female ratio; rather, these variables were accounted for during data analysis (see Data Analysis section below). On postnatal day 1 (PND1), animals from each litter were randomly allocated to the ELS or control (no ELS) condition. ELS allocated litters underwent maternal separation procedures (detailed below) between PND2-14. On PND21, animals were weaned and separated into same-sex housing, with 2 animals/cage (41.5 × 28 × 22 cm cages; Mascot Wire Works, Sydney). Food (Rat and Mouse Pellets, Glen Forest, Western Australia) and water were available *ad libitum* and rats were maintained on a 12-h light (0600–1800): 12 h dark cycle. Temperature was maintained at 20 ± 2°C and humidity was kept at 34 ± 2%.

EARLY LIFE STRESS

An overview of the experimental design is outlined in **Figure 1**. The maternal separation procedure was performed as per previously published procedures in our laboratory (Nakamura et al., 2011), that were based on earlier studies (Plotsky and Meaney, 1993). Briefly, from PND2-14, litters in the ELS condition were removed from their home cage and individually placed in clear separation containers (13 × 13 × 7 cm) in an alternate temperature controlled room (30–34°C) for 3 h each day, from 0900 to 1200 h. Pups in the control condition remained undisturbed during this period except for weekly weighing. Bedding was left undisturbed for one week after birth, after which it was changed on a weekly basis.

EXERCISE

A subgroup of animals exposed to ELS (males $n = 6$; females $n = 9$) was allowed access to a running wheel located in a separate room between PND40–70 (85 × 7.5 cm, 94 × 12 cm; Transoniq; for 1hr/day, 5days/week between 1800 and 2100 h). Only animals exposed to ELS were given access to exercise wheels, as pilot studies indicated that wheel running had no behavioral consequences for animals not exposed to ELS. (i.e., ELS+exercise group did not differ significantly from no-ELS+exercise; see Supplementary Material 1). A rotation counter attached to each wheel quantified distance traveled. Food intake was estimated across all groups during the exercise period by weighing food daily and dividing the change in food weight by the number of animals per cage.

ADULT STRESS EXPOSURE

Pilot studies revealed that maternal separation had no effect on open field (OF) behavior in the absence of an additional stressor in adulthood (see Supplementary Material 2). As such, between PND75-79, all animals were exposed to 30 min restraint stress prior to behavioral testing. Animals were removed from their home cage and were placed inside a soft wire mesh restrainer (25 × 20 cm) that was folded around the animal and secured with butterfly clips. This procedure has been previously demonstrated to produce a pattern of Fos-activity centered on amygdaloid and brainstem catecholamine nuclei that is distinct from physical stressors (Dayas et al., 1999). Females were tested only in the diestrous phase, monitored using a rat vaginal impedance device (Muromachi Kikai, Tokyo), as described elsewhere (Walker et al., 2010).

BEHAVIORAL TESTING

Both OF and EPM testing was conducted in darkness using infrared lighting. Time and event data for both apparatuses was recorded using a computer-automated behavioral tracking system (Motion Mensura Ltd., Australia). Immediately following restraint stress, animals were placed in a square 1 × 1 m open field task apparatus enclosed by 40 cm high walls for 10 min. Exploratory variables measured were total distance traveled and time in immobility. Immediately following OF testing, approximately half of the animals (males $n = 18$; females $n = 24$) were tested on the EPM apparatus whilst the remaining animals (males $n = 16$; females $n = 15$) were returned to their home cage. Animals were transferred to a separate room where they were placed on an EPM apparatus. The EPM was painted black, and consisted of two open and two closed arms (45 cm length × 10 cm width) as well as a central square (10 × 10 cm). The primary measures on this assay included the time spent in the open arms,

FIGURE 1 | A schematic illustration of the experimental design. Neonatal treatment consisted of either early life stress (maternal separation) for 3hrs/day from postnatal days (PND) 2-14, or no early life stress. A subgroup of animals was given access to running wheels for 1 hr/day, 5days/week from PND40-70. All animals were subjected to restraint stress in adulthood (PND75-79) for 30 min. Immediately following restraint, animals underwent behavioral testing in the open field test (10 min) and elevated plus maze (5 min). Ninety minutes following restraint stress, animals were euthanized and brains collected.

an index of anxiety-related behavior, and the number of closed arm entries, a measure of overall locomotor activity (Richardson et al., 2006). We also measured number of entries into the open arms and center square, as well as latency to enter the open and closed arms. Importantly, EPM-challenged animals did not differ from non-EPM-challenged animals in terms of Fos-protein expression in any of the regions studied, and therefore data from these animals were combined.

BRAIN TISSUE HARVESTING AND IMMUNOHISTOCHEMISTRY

Two hours following the initiation of restraint stress (1hr 20min following OF; 1hr 15min following EPM), rats were deeply anesthetized with sodium pentobarbitone (200 mg/kg; i.p.; Virbac, Australia). Animals were then perfused with 200 mL of 0.1 M Phosphate Buffered Saline followed by 500 mL of 4% paraformaldehyde (pH 9.5). Brains were removed and postfixed in 4% paraformaldehyde at 4°C overnight and then stored in 12.5% sucrose until sectioning. Serial rostral forebrain (40-μm) and caudal midbrain (50-μm) sections were cut using a freezing microtome (Leica Microsystems, SM2000R) and a 1-in-4 series of all sections were processed for immunohistochemical detection of Fos-protein (72 h, 1:5000, rabbit polyclonal, Santa Cruz Biotechnology, CA, USA) as described previously in detail (Smith and Day, 1993; Dayas et al., 2008). Hypothalamic sections were dual-labeled for orexin A (48 h, 1:15000, Orexin A antibody, goat polyclonal, Santa Cruz Biotechnology) or in the case of ventral tegmental area (VTA) sections, tyrosine hydroxylase (TH; 48 h, 1:10000, TH antibody, mouse polyclonal, Millipore). An equal number of animals from each treatment group were included in each individual immunohistochemistry run.

Bilateral counts of single-labeled Fos-positive cells were made in the perifornical area (PFA) and lateral hypothalamus (LH; bregma −2.28 to −3.24), paraventricular thalamus (PVT; −2.76 to −3.24) and medial parvocellular PVN (mpPVN; −1.46 to −1.94). Fos-only cell counts in the PVN and PVT were quantified using Metamorph Imaging System Software (Version 7.5; Molecular Devices Analytical Technologies) at 10× total magnification (Olympus CX40). The number of Fos-positive cells was determined by creating a region of interest around each structure and a thresholding procedure was used to quantify Fos expression. Counts of Fos-positive orexin neurons in the LH and Fos-positive TH cells in the VTA (−5.30 to −5.94) were made by one observer blind to treatment using a 20× objective (Olympus CX40). In the LH, cell counts were made in the PFA and the LH divisions, as these sections have previously been shown to contain the highest concentration of orexin neurons (Dayas et al., 2008). The PFA was defined as the area surrounding the fornix and the LH was defined as the area from the lateral side of the PFA to the optic tract (Laorden et al., 2012). Cells in the VTA were quantified in the parabrachial pigmented nucleus (PBP) region of the VTA. All brain coordinates were based on the Paxinos and Watson atlas (Paxinos and Watson, 2007).

DATA ANALYSIS

All statistical analyses were conducted using IBM SPSS V19. Male and female animals were analyzed separately. ANCOVA revealed no significant effect of litter size and male to female ratio for all comparisons. Body weight of treatment groups was compared on PND72 using a one-way between-subjects ANOVA. Food intake and behavioral data were compared across treatment groups using a one-way between-subjects ANOVA and subsequent least significant differences (LSD) *post-hoc* analyses where appropriate. For immunohistochemical analyses, all cell counts were averaged across each animal for each rostrocaudal level of each brain region examined. To minimize the effects of variability across multiple immunohistochemistry runs, counts for each treatment group were calculated as a fold change relative to control animals processed in the same run. These fold changes were averaged across the rostral-caudal extent of each brain region and were compared across groups using one-way ANOVAs. These analyses were followed by LSD *post-hoc* analyses where appropriate. An alpha value of 0.05 was adopted for all statistical tests. All figures depict means and standard errors.

RESULTS

EFFECT OF ELS ON BODY WEIGHT AND FOOD INTAKE

On PND72, male animals from each treatment group did not differ significantly in terms of their body weight [$F_{(2, 31)} = 2.366$, $p = 0.106$], or food intake across the experimental period [$F_{(2, 14)} = 2.554$, $p = 0.113$]. Similarly, body weight of females was indistinguishable between treatment groups [$F_{(2, 36)} = 0.026$, $p = 0.975$] as was their food intake [$F_{(2, 20)} = 0.302$, $p = 0.743$]. Interestingly, wheel rotations were on average approximately three times higher in females than male animals in each exercise session [$F_{(1, 13)} = 19.429$, $p < 0.001$; **Figure 2**].

ELS WAS ASSOCIATED WITH A REDUCED PERCENTAGE OF FOS-POSITIVE OREXIN CELLS AFTER PSYCHOLOGICAL STRESS: PROTECTIVE EFFECT OF EXERCISE ONLY IN MALES

In male rats there was no effect of treatment on the number of orexin immunoreactive cells in either the PFA or LH subdivisions of the hypothalamus [$F_{(2, 18)} = 0.292$, $p = 0.750$; $F_{(2, 18)} = 1.648$, $p = 0.220$ respectively, data not shown]. To assess the effect of ELS on the reactivity of orexin neurons to stress in adulthood, we quantified the percentage of orexin cells expressing Fos-protein following psychological stress. ANOVA revealed a significant effect of treatment on the percentage of orexin cells expressing Fos protein in the PFA [$F_{(2, 18)} = 17.646$, $p < 0.001$], and a trend toward significance in the LH [$F_{(2, 18)} = 3.248$, $p = 0.062$]. *Post-hoc* analyses revealed that ELS animals displayed a significantly lower percentage of orexin neurons that expressed Fos-protein after psychological stress compared to controls in the PFA ($p = 0.002$). Interestingly, ELS animals given access to running wheels displayed a pattern of Fos/orexin immunoreactivity in the PFA that was significantly greater than that of other treatment groups ($p = 0.042$ compared to controls, $p < 0.001$ compared to ELS; **Figure 3**).

Similar to males, orexin cell numbers did not differ across treatment groups in female rats in both the PFA and LH [$F_{(2, 18)} = 0.141$, $p = 0.87$; $F_{(2, 18)} = 0.166$, $p = 0.849$, respectively; data not shown]. There was a significant main effect of treatment on the percentage of orexin cells that displayed Fos-like immunoreactivity in response to restraint stress in the PFA [$F_{(2, 18)} = 26.907$, $p < 0.001$] and LH [$F_{(2, 18)} = 14.292$, $p <$

FIGURE 2 | Effect of early life stress (ELS) on body weight and food intake; and sex differences in wheel running. There was no effect of treatment on body weight at postnatal day 72 in both male and female rats. Males: No ELS: $n = 13$; ELS: $n = 15$; ELS + Ex: $n = 6$. Females: No ELS: $n = 16$; ELS: $n = 14$; ELS + Ex: $n = 9$ **(A)**. Similarly, ELS had no effect on food intake. Males: No ELS: $n = 6$; ELS: $n = 6$; ELS + Ex: $n = 5$. Females: No ELS: $n = 7$; ELS: $n = 7$; ELS + Ex: $n = 9$ **(B)**. Female rats engaged in significantly greater amounts of wheel running per day compared to male rats. Males: $n = 6$; Females: $n = 9$ **(C)**. $^{***}p < 0.001$.

0.001]. Consistent with male animals, *post-hoc* analyses showed that ELS females exhibited a significantly lower percentage of Fos-positive orexin cells compared to control animals in the PFA ($p = 0.018$) and a similar trend in the LH ($p = 0.094$). In contrast to males however, access to running wheels tended to exacerbate the effect of treatment on orexin cell reactivity as assessed by Fos-labeling in the PFA ($p < 0.001$ compared to controls and ELS) and LH ($p < 0.001$ compared to controls, $p < 0.01$ compared to ELS; **Figure 3**).

ELS WAS ASSOCIATED WITH A REDUCTION IN FOS-PROTEIN EXPRESSION IN PVN AND PVT NEURONS FOLLOWING PSYCHOLOGICAL STRESS: PROTECTIVE EFFECT OF EXERCISE IN MALE BUT NOT FEMALE RATS

In addition to orexin neurons we assessed the level of Fos-like immunoreactivity in the VTA, PVN and PVT following restraint stress in adulthood. In males, the percentage of Fos-positive TH cells in the VTA did not differ significantly between treatment groups [$F_{(2, 15)} = 1.369$, $p = 0.284$; **Figure 4**]. There was a significant main effect of treatment on Fos-immunoreactivty in the PVN [$F_{(2, 15)} = 9.316$, $p = 0.002$], with *post-hoc* analyses revealing a significant reduction in Fos-positive cells in ELS animals compared to controls ($p = 0.008$). Access to voluntary exercise significantly increased the number of Fos-positive PVN cells compared to ELS-exposed animals ($p < 0.001$). There was no significant difference between exercised males and controls in this region ($p = 0.287$; **Figure 4**). In the PVT, there was a significant main effect of treatment on Fos-positive cells [$F_{(2, 19)} = 5.248$, $p = 0.015$]. *Post-hoc* analyses revealed a significant increase in Fos-immunoreactivity in the PVT of animals given access to running wheels ($p = 0.023$ compared to controls, and $p = 0.006$ compared to ELS; **Figure 4**). No significant difference was found in the number of Fos-positive cells in the ELS group compared to controls in this region ($p = 0.602$).

In females, there was no significant main effect of treatment on the number of TH-positive cells that expressed Fos-protein [$F_{(2, 18)} = 1.415$, $p = 0.269$; **Figure 4**]. In the PVN, ANOVA revealed a significant main effect of treatment on the number of Fos-positive cells [$F_{(2, 19)} = 8.27$, $p = 0.003$]. *Post-hoc* analyses revealed no significant difference between controls and ELS ($p = 0.152$). However, access to running wheels significantly reduced the number of Fos-positive PVN cells ($p < 0.001$ compared to controls, $p = 0.016$ compared to ELS; **Figure 4**). In the PVT, there was a significant main effect of treatment on Fos-protein expression [$F_{(2, 19)} = 6.409$, $p = 0.008$]. *Post-hoc* analyses revealed that there was no significant difference in the number of Fos-positive cells in ELS animals compared to controls ($p = 0.156$). A significant reduction in Fos-positive PVT cells was observed in rats given access to running wheels ($p = 0.002$ compared to controls, $p = 0.041$ compared to ELS; **Figure 4**).

ELS ANIMALS HAD LOWER EXPLORATORY BEHAVIOR IN THE OPEN FIELD FOLLOWING PSYCHOLOGICAL STRESS IN ADULTHOOD: PROTECTIVE EFFECT OF EXERCISE IN MALE BUT NOT FEMALE RATS

In males, one-way ANOVA revealed a main effect of treatment on the distance traveled in the OF [$F_{(2, 31)} = 2.66$, $p = 0.043$]. *Post-hoc* comparisons revealed that ELS-exposed animals traveled significantly less distance compared to controls ($p = 0.026$). This effect was reversed when ELS animals were given access to voluntary exercise throughout adolescence ($p = 0.044$, compared to ELS group; **Figure 5**). Analyses also showed a trend toward the ELS group exhibiting increased time spent in immobility as compared to the control group, with this effect again being reversed by exercise intervention [$F_{(2, 31)} = 2.21$, $p = 0.063$, data not shown).

In females, there was a significant main effect of treatment in terms of distance traveled [$F_{(2, 36)} = 7.13$, $p = 0.001$]. Similar to males, maternally separated animals exhibited locomotor hypoactivity in the OF when compared to controls ($p = 0.007$). In contrast to males, this effect was not reversed by voluntary exercise, but in fact was exaggerated ($p < 0.001$, compared to controls; **Figure 5**). This same trend was observed in terms of time spent in immobility [$F_{(2, 36)} = 7.44$, $p = 0.001$] with ELS females spending significantly more time in immobility compared to controls ($p = 0.01$). Time spent in immobility was exaggerated in exercised females ($p < 0.001$ compared to controls, data not shown).

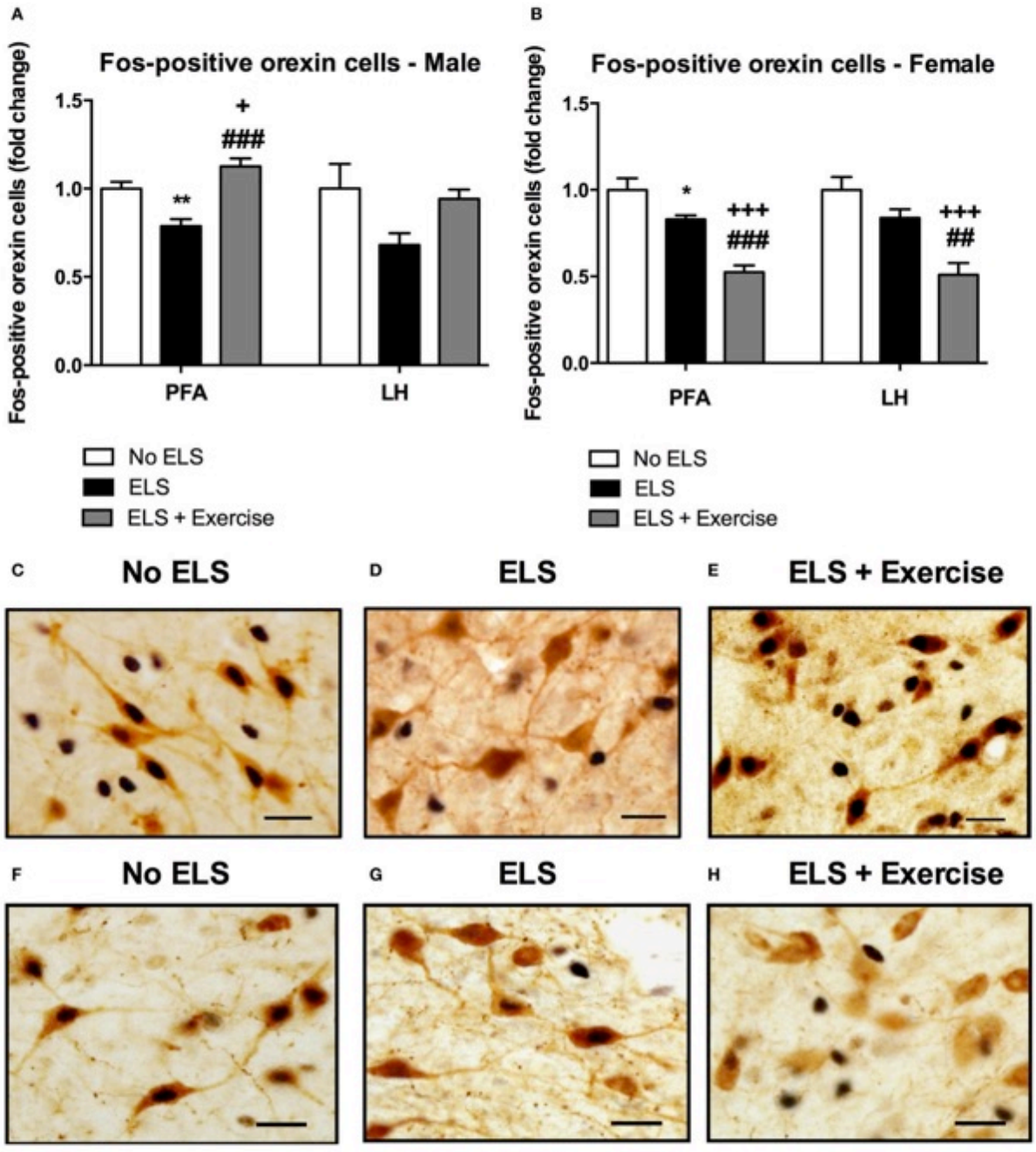

FIGURE 3 | Early life stress (ELS) was associated with a decrease in the percentage of Fos-positive orexin cells in both male and female rats: Wheel running protected against these effects in males, but exaggerated these effects in females. The percentage of Fos-positive orexin cells in the PFA of the hypothalamus was significantly lower in male ELS rats compared to controls. Wheel running was protective against the effects of ELS in this region. This trend was also observed in the LH but failed to reach significance ($p = 0.06$; **A**). As in male animals, ELS-exposed females exhibited a reduced percentage of Fos-positive orexin cells in the PFA. In contrast to males, wheel running exacerbated these effects in female rats **(B)**. A similar trend was observed in the LH **(B)**. Photomicrographs of coronal sections of the PFA of the hypothalamus immunolabeled for Fos-protein and orexin in males **(C,D,E)** and females **(F,G,H)**. Males: No ELS: $n = 7$; ELS: $n = 9$; ELS + Ex: $n = 6$. Females: No ELS: $n = 7$; ELS: $n = 8$, ELS + Ex: $n = 7$. $^{*}p < 0.05$ vs. No ELS, $^{**}p < 0.01$ vs. No ELS, $^{+}p < 0.05$ vs. No ELS, $^{+++}p < 0.001$ vs. No ELS, $^{\#\#}p < 0.01$ vs. ELS, $^{\#\#\#}p < 0.001$ vs. ELS, scale bar, 20 μm.

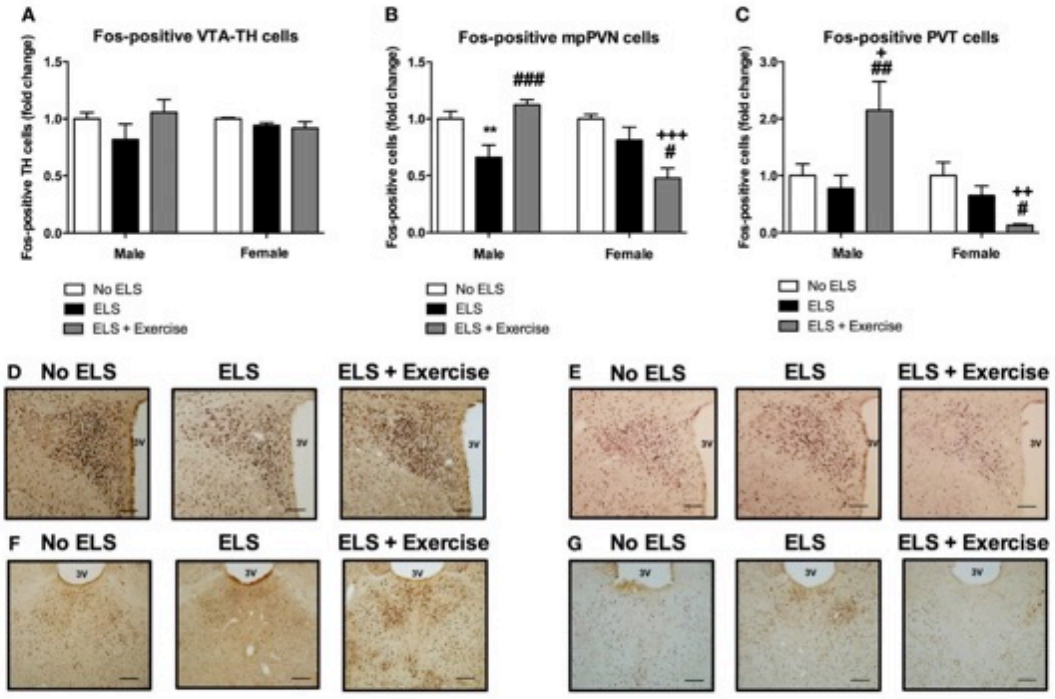

FIGURE 4 | Early life stress (ELS) was associated with a decrease in Fos-immunoreactivity in mpPVN and PVT neurons: Protective effect of exercise in male but not female rats. ELS resulted in a non-significant reduction in the percentage of Fos-positive TH cells in the VTA in male rats. Wheel running appeared to be protective against these effects. No ELS: $n = 6$; ELS: $n = 6$, ELS + Ex: $n = 6$ **(A)**. In females, there was no effect of ELS or exercise on the percentage of Fos-positive TH cells in the VTA. No ELS: $n = 7$; ELS: $n = 7$; ELS + Ex: $n = 7$ **(A)**. In the PVN, there was an ELS-induced reduction in Fos-positive cells in males and this effect was reversed by wheel running. There was no effect of ELS on the number of Fos-positive cells in the PVT in males however, wheel running was protective against the effects of ELS. No ELS: $n = 7$; ELS $n = 9$; ELS + Ex: $n = 6$ **(B,C)**. In females, there was no effect of ELS on the number of Fos-positive cells in the PVN and PVT however, wheel running did exacerbate the effects of ELS. No ELS: $n = 7$; ELS: $n = 8$; ELS + Ex: $n = 7$ **(B,C)**. Coronal sections of the PVN (males: **D**; females: **E**) and PVT (males: **F**; females: **G**) immunolabeled for Fos-protein, scale bar 100 μm. $^{**}p < 0.01$ vs. No ELS, $^{+}p < 0.05$ vs. No ELS, $^{++}p < 0.01$ vs. No ELS, $^{+++}p < 0.001$ vs. No ELS, $^{\#}p < 0.05$ vs. ELS, $^{\#\#}p < 0.01$ vs. ELS, $^{\#\#\#}p < 0.001$ vs. ELS.

FIGURE 5 | Early life stress (ELS) is associated with reduced locomotor activity in the open field task: Protective effects of exercise in males, but not in females. In both males and females, ELS was associated with a significant reduction in the distance traveled in the open field. Wheel running protected against this effect in male rats, but exacerbated the ELS effect in female rats **(A)**. In both male and female rats, there was no effect of treatment or wheel running on the number of open **(B)** or closed **(C)** arm entries across in the elevated plus maze. Males: No ELS: $n = 13$; ELS: $n = 15$; ELS + Ex: $n = 6$. Females: No ELS: $n = 16$; ELS: $n = 14$; ELS + Ex: $n = 9$. $^{*}p < 0.05$ vs. No ELS, $^{**}p < 0.01$ vs. No ELS, $^{++}p < 0.01$ vs. No ELS, $^{\#}p < 0.05$ vs. ELS.

With respect to the EPM, there was no effect of treatment on the number of open [$F_{(2, 15)} = 0.382$, $p = 0.689$] or closed [$F_{(2, 15)} = 1.624$, $p = 0.230$] arm entries in males. Similarly, in females there was no difference between treatment groups on open [$F_{(2, 21)} = 0.617$, $p = 0.549$] or closed arm entries [$F_{(2, 21)} = 0.040$, $p = 0.961$; **Figure 5**]. Additionally, there was no effect of treatment on the duration spent in the open arms, closed arms or the center square, or the latency to enter the open or closed arms in either males or females (p's > 0.05).

DISCUSSION

In the present study we report that ELS-exposed male and female rats exhibited a "hypoactive" orexin cell response to restraint stress, as assessed by Fos-like immunoreactivity, particularly in the PFA. Notably, both male and female animals exposed to ELS displayed reduced exploratory behavior on the OF following restraint stress. Interestingly, the ability of exercise to ameliorate ELS-induced deficits was strongly sex-dependent. A similar level of sex-specificity was also seen in brain regions that are known to respond to orexin innervation. Together these results not only highlight the profound effect that ELS has on orexin function in adulthood but also the positive effects of exercise on this deficit and how this differs across sexes.

The primary aim of this study was to assess the impact of ELS on subsequent orexin cell reactivity to psychological stress in adulthood. Due to known sex differences in neural responses to stress, we examined the degree to which orexin cells had become activated by quantifying the number of orexin cells that were Fos-positive following psychological stress in adulthood in both male and female rats. Using this well-characterized strategy, orexin function in both males and females that were exposed to ELS was substantially lower than non-ELS controls. ELS-exposed animals also exhibited significantly lower activity in the OF. These findings are interesting in light of recent findings from other preclinical studies that have shown that chronic stress results in reduced orexin system function and increased depressive-like behavior (Lutter et al., 2008; Nocjar et al., 2012). Further, recent human studies have reported an inverse relationship between CSF orexin peptide levels and symptoms of depression (Brundin et al., 2007, 2009). With these findings in mind, it is possible that in our study, reduced orexin activity induced by ELS resulted in a depressive-like behavioral state that manifested as reduced exploratory behavior. Future studies should assess whether these changes in orexin cell function also manifest as deficits in motivated behavior on behavioral assays such as the sucrose preference test and/or forced swim test. Further investigation is also warranted to understand the relevance of our observation that orexin hypoactivity was more pronounced in the PFA compared to the LH, as separate functions have been ascribed to these populations (stress reactivity and reward-seeking, respectively; Harris and Aston-Jones, 2006).

Perhaps the most striking observation of the present study was that behavioral deficits associated with ELS were not observed in male rats allowed access to voluntary exercise. These findings are consistent with previous studies demonstrating that voluntary wheel running protects against the expression of anxiety-like behavior in adult male rats exposed to maternal separation stress (Maniam and Morris, 2010) or footshock stress in adulthood (Greenwood et al., 2013). Given that there was a significant "wash-out" period between wheel running and restraint, it is likely that exercise reversed ELS-induced changes in LH-orexin circuit function rather than prevented the acute effects of restraint. However, we acknowledge further tests are required to address this issue. With respect to the sex-specific effects we observed, our findings are in line with those of Brocardo et al. (2012) who showed that voluntary exercise had no effect on the expression of anxiety- and depression-like behaviors in female rats exposed to ethanol in early life, despite this intervention having protective effects in males (Brocardo et al., 2012). Similarly, findings from the addiction field have yielded differential effects of voluntary exercise on drug-related behaviors in males and females, despite being exposed to identical exercise regimes (Smith et al., 2008; Ehringer et al., 2009; Thanos et al., 2010). Our findings that exercise actually tended to exacerbate ELS-induced orexin and behavioral changes in females perhaps points to the possibility that the increased wheel running observed in females was actually stress provoking. Exercise-induced corticosterone secretion may have subsequently influenced orexin cell responsivity (Ford et al., 2005). These findings point to the need for a greater understanding of how exercise conditions (type, intensity, duration) can be modified to produce beneficial effects in both sexes.

Interestingly, we observed a similar pattern of reactivity in key orexinergic targets, including the mpPVN, PVT, and VTA dopamine neurons. With respect to the mpPVN, there is now considerable evidence that orexin directly modulates the neuroendocrine response to stress. The mpPVN is densely innervated by orexinergic terminals and mainly expresses orexin receptor 2 (Peyron et al., 1998; Trivedi et al., 1998). Further, central administration of orexin-A induces Fos-protein expression in corticotropin releasing factor (CRF)-expressing cells in the mpPVN (Sakamoto et al., 2004) and increases plasma corticosterone and adrenocorticoptropin hormone (ACTH) levels (Ida et al., 2000; Kuru et al., 2000). Whilst we did not directly measure HPA-axis activity, maternal separation stress has previously been shown to be associated with impaired mpPVN and HPA-axis responsivity to stress in adulthood (Plotsky and Meaney, 1993; Ladd et al., 2000; Daniels et al., 2004). Further studies are required to assess whether this impairment is directly associated with the reduction in orexin function observed here. With respect to the PVT, this region is also known to be densely innervated by orexin terminals (Kirouac et al., 2005) and contains high densities of both orexin receptors (Marcus et al., 2001). Orexin signaling in the PVT has recently been shown to be important for both the neuroendocrine response to stress (Heydendael et al., 2012) and the expression of stress-related behaviors (Li et al., 2010a,b; James and Dayas, 2013; Yeoh et al., 2014). Further, stimulation of the PVT can modulate dopamine release in the nucleus accumbens (NAC; Jones et al., 1989; Parsons et al., 2007). Reduced PVT signaling in ELS animals may therefore contribute to reduced striatal dopamine release, an outcome consistent with previous studies showing that hypoactivity of the VTA dopamine-NAC projection is causally linked to depressive-like behavior following social defeat (Berton et al., 2006).

One caveat of our experimental design is that OF and EPM testing were performed after restraint stress. While we attribute

the pattern of Fos-protein expression observed as a response to restraint stress, given the time course typically required for maximal Fos-protein induction (2 h; Kovács, 1998), we cannot exclude the possibility that OF and EPM testing also influenced the expression of this immediate early gene. Regardless, these challenges are typically regarded as psychological stressors, which produce similar patterns of Fos-protein expression in stress-sensitive brain regions as restraint stress (Dayas et al., 2001). Further, no differences were observed in terms of Fos-protein expression amongst EPM-challenged vs. non-EPM-challenged animals, suggesting that behavioral testing did not have any confounding effects on Fos expression. It is also important to note that a previous report failed to observe an increase in Fos-protein expression in orexin-positive neurons in response to restraint (Furlong et al., 2009). These experiments however, were carried out in animals with no prior stress exposure, and the effects observed in our study may reflect a more important role for orexin signaling in stress reactivity in chronically stressed animals.

In summary, the present study provides novel evidence that the orexin system's response to adult stress is altered by ELS. Identical effects of ELS on orexin cell activity in stressed adults were observed in the PFA of male and female rats. These data are consistent with recent clinical evidence indicating that vulnerability to stress-related mood disorders is linked with orexin system hypofunction (Brundin et al., 2007, 2009). We also show that exercise was protective against both the behavioral (OF activity) and neural effects of ELS in male rats, suggesting that the beneficial effects of exercise on stress-related behavior is associated with a "normalization" of orexin function and that, under some conditions, the orexin system can be modified by non-pharmacological methods. Surprisingly, female rats exhibited significantly greater deficits in orexin function following wheel running, suggesting that while the effects of ELS on orexin function are similar across sexes, future studies will need to consider alternative approaches to recover orexin function in female rats. These findings extend recent studies showing that the orexin system is highly plastic and is readily modified by environmental events (Yeoh et al., 2012). More broadly, this study highlights the importance of studying sex-based differences in stress-related pathology (Clayton and Collins, 2014).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnbeh.2014.00244/abstract

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CHAPTER TWO

Role of ventral subiculum in context-induced relapse to alcohol seeking after punishment-imposed abstinence.

Marchant, N. J., **Campbell, E. J.,** Whitaker, L. R., Harvey, B. K., Kaganovsky, K., Adhikary, S., Hope, B. T., Heins, R. C., Prisinzano, T. E., Vardy, E., Bonci, A., Bossert, J. M., & Shaham, Y.

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DEPARTMENT OF HEALTH & HUMAN SERVICES

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National Institute on Drug Abuse
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8 March 2016

To whom it may concern,

Erin J. Campbell was a second author on the publication “Role of ventral subiculum in context-induced relapse to alcohol seeking after punishment-imposed abstinence” (J. Neurosci, 2016) in my laboratory at the National Institute on Drug Abuse, Baltimore, USA. Erin was conducting this research under a travel scholarship from the Hunter Medical Research Institute (HMRI) and the University of Newcastle, NSW, Australia.

I confirm that her contribution to this manuscript included performing the experiments, analyzing the data, and writing the manuscript.

As laboratory head, I attest on behalf of all authors including myself, to the contribution of Erin to this publication.

Sincerely,

Yavin Shaham, Ph.D.
Branch Chief and Senior Investigator
Intramural Research Program/NIDA/NIH

June, 2016

PROFESSOR ROBERT CALLISTER
Deputy Head of Faculty (Research and Research Training)

Systems/Circuits

Role of Ventral Subiculum in Context-Induced Relapse to Alcohol Seeking after Punishment-Imposed Abstinence

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In many human alcoholics, abstinence is self-imposed because of the negative consequences of excessive alcohol use, and relapse is often triggered by exposure to environmental contexts associated with prior alcohol drinking. We recently developed a rat model of this human condition in which we train alcohol-preferring P rats to self-administer alcohol in one context (A), punish the alcohol-reinforced responding in a different context (B), and then test for relapse to alcohol seeking in Contexts A and B without alcohol or shock. Here, we studied the role of projections to nucleus accumbens (NAc) shell from ventral subiculum (vSub), basolateral amygdala, paraventricular thalamus, and ventral medial prefrontal cortex in context-induced relapse after punishment-imposed abstinence. First, we measured double-labeling of the neuronal activity marker Fos with the retrograde tracer cholera toxin subunit B (injected in NAc shell) and demonstrated that context-induced relapse is associated with selective activation of the vSub→NAc shell projection. Next, we reversibly inactivated the vSub with GABA receptor agonists (muscimol+baclofen) before the context-induced relapse tests and provided evidence for a causal role of vSub in this relapse. Finally, we used a dual-virus approach to restrict expression of the inhibitory κ opioid-receptor based DREADD (KORD) in vSub→NAc shell projection neurons. We found that systemic injections of the KORD agonist salvinorin B, which selectively inhibits KORD-expressing neurons, decreased context-induced relapse to alcohol seeking. Our results demonstrate a critical role of vSub in context-induced relapse after punishment-imposed abstinence and further suggest a role of the vSub→NAc projection in this relapse.

Key words: alcohol; chemogenetics; nucleus accumbens; punishment; relapse; ventral subiculum

Significance Statement

In many human alcoholics, abstinence is self-imposed because of the negative consequences of excessive use, and relapse is often triggered by exposure to environmental contexts associated with prior alcohol use. Until recently, an animal model of this human condition did not exist. We developed a rat model of this human condition in which we train alcohol-preferring P rats to self-administer alcohol in one context (A), punish the alcohol-reinforced responding in a different context (B), and test for relapse to alcohol seeking in Contexts A and B. Here, we used neuroanatomical, neuropharmacological, and chemogenetic methods to demonstrate a role of ventral subiculum and potentially its projections to nucleus accumbens in context-induced relapse after punishment-imposed abstinence.

Introduction

In abstinent alcoholics, exposure to environments previously associated with alcohol use often causes relapse (Wikler, 1973; Collins and Brandon, 2002). We and others have modeled this phenomenon in rats using the ABA renewal model (Bouton and Bolles, 1979). In this model, rats are first trained to self-administer a drug in one context (A). Next, they are given oper-

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ant extinction training in a different context (B), leading to cessation of drug seeking. During testing, renewal (or context-induced reinstatement) of drug seeking is observed when the rat returns to the original training Context A (Crombag and Shaham, 2002; Hamlin et al., 2007). Over the last decade, studies using this model have led to the identification of mechanisms of context-induced reinstatement of drug seeking after extinction (Fuchs et al., 2008a; McNally, 2014; Marchant et al., 2015).

However, a limitation of extinction-based models is the use of experimenter-imposed operant extinction to achieve abstinence (Epstein and Preston, 2003). This manipulation is different from human abstinence, which is typically self-imposed because of the adverse consequences of drug use (Klingemann, 1991; Blume et al., 2006). We recently developed a variation of the ABA renewal model (Marchant et al., 2013; see also Bouton and Schepers, 2015), where alcohol intake is suppressed by adverse consequences in Context B (punishment). We observed context-induced relapse to alcohol seeking when rats were tested in Context A after punishment-imposed abstinence in Context B (Marchant et al., 2013).

The nucleus accumbens (NAc) shell plays a critical role in context-induced reinstatement of drug seeking after extinction (Bossert et al., 2013). We recently found that blockade of NAc shell dopamine D1 receptors (D1R), which decreases striatal neuronal activity (Girault et al., 2007), blocks context-induced relapse to alcohol seeking after punishment (Marchant and Kaganovsky, 2015). In NAc shell, neuronal activity is dependent on synergistic activation of D1R and glutamate receptors (O'Donnell, 2003). Inhibition of glutamate transmission in NAc shell decreases context-induced reinstatement of heroin and cocaine seeking after extinction (Bossert et al., 2006; Xie et al., 2012); however, local injections of glutamate receptor antagonists also cause reinstatement of alcoholic beer seeking after extinction (Millan and McNally, 2011). Additionally, neuroanatomical disconnection of the ventral subiculum (vSub)→NAc shell or vSub inactivation decreases context-induced reinstatement of heroin seeking (Bossert and Stern, 2014; Bossert et al., 2015). There is also evidence that the two other glutamatergic projections from the basolateral amygdala (BLA) and paraventricular thalamus (PVT) to NAc shell (Voorn et al., 2004) contribute to context-induced reinstatement after extinction (Fuchs et al., 2005; Hamlin et al., 2009; Marinelli et al., 2010).

Here we studied the role of the projections to NAc shell in context-induced relapse to alcohol seeking after punishment-imposed abstinence. We first combined retrograde tracing, via cholera toxin subunit B (CTb) injections into NAc shell, with detection of the neuronal activity marker Fos (Morgan and Curran, 1991) to determine relapse-associated activity in vSub, PVT, BLA, and ventral medial prefrontal cortex (vmPFC) neurons projecting to NAc shell. We found that context-induced relapse was associated with selective activation of the vSub→NAc shell projection. Next, we determined the causal role of vSub in context-induced relapse by showing that reversible inactivation of vSub by GABAa+GABAb receptor agonists (muscimol+baclofen) (McFarland and Kalivas, 2001) decreased this form of relapse. Finally, we used DREADD technology (Armbruster et al., 2007) to selectively inhibit the vSub→NAc shell projection during the relapse tests. We used a dual-virus approach (Nair et al., 2013; Boender et al., 2014; Marchant et al., 2016), by injecting AAV5-Cre into NAc shell; and in the same rat, we injected cre-inducible AAV encoding κ-opioid receptor-based DREADD (KORD) (Vardy et al., 2015) into vSub to selectively express KORD in vSub→NAc shell projection neurons. We found that systemic injection of the designer drug salvinorin B (SalB), which inhibits neuronal activity of KORD-expressing cells (Vardy et al., 2015; Marchant et al., 2016), decreased context-induced relapse after punishment-imposed abstinence in rats expressing KORD in vSub.

The authors declare no competing financial interests.

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Materials and Methods

Subjects and apparatus

We received male alcohol-preferring P rats (~30-d-old, total $n = 115$) from Indiana University Medical Centre and housed them singly under a reverse 12 h light/dark cycle (lights off 0800) with food and water available *ad libitum*. We performed all experiments in accordance to the *Guide for the Care and Use of Laboratory Animals* (Ed 8), and all protocols were approved by the Animal Care and Use Committee. We excluded a total of 24 rats due to lost head cap ($n = 8$), sickness or weight loss in excess of 30 g after intracranial injections ($n = 1$), misplaced cannula ($n = 2$), complications during surgery ($n = 7$), or low KORD ($n = 3$) or hM3D ($n = 3$ expression). We used standard operant chambers (Med Associates) enclosed in a ventilated sound-attenuating cubicle illuminated by a house light for alcohol self-administration. Each chamber was equipped with one retractable lever (designated as "active") and one nonretractable lever (designated as "inactive"). The grid floors were connected to shockers. Active lever presses resulted in the delivery of 20% ethanol (0.1 ml/delivery) into the receptacle via a 12-gauge blunt needle connected to a 60 ml syringe controlled by a Razel pump. We manipulated Contexts A and B as previously described (Marchant et al., 2013): grid width (narrow/wide), illumination level (white/red house light), background noise (fan on/off), and background cues (food container present/absent within the operant chamber, cabinet doors closed/open).

Behavioral procedure (four phases)

Phase 1: Home-cage alcohol intake. We used an intermittent access (3–4 times/week) alcohol procedure (Wise, 1973; Simms et al., 2008) in which rats received 12 × 24 h sessions of access to one bottle of 20% alcohol and one water bottle. We prepared alcohol solutions in tap water from 100% (v/v) ethanol in standard rat water bottles. Daily sessions began at 0900. After 24 h, we replaced the alcohol bottle with a second water bottle for the subsequent 24–48 h alcohol-free days. The following day, the second water bottle was replaced with the 20% alcohol bottle, and the location of the alcohol bottle was alternated from the previous session. Total alcohol consumption in grams was calculated for each session, using the weight difference between the beginning and end of the session, minus 2 g for spillage, multiplied by 0.97 (density of 20% ethanol).

Phase 2: Operant self-administration: Context A. We gave all rats two 2 h magazine-training sessions where 0.1 ml of alcohol was delivered noncontingently every 5 min and paired with a 2 s tone-light cue. Next, we trained rats for six 2 h self-administration sessions under a fixed-ratio 1 (FR-1) schedule of reinforcement where an active lever press resulted in the delivery of 0.1 ml of 20% alcohol paired with a 2 s tone-light cue. This was followed by a 20 s timeout period where lever presses were recorded but not reinforced. The initiation of each session was signaled by the illumination of the house light and the insertion of the active lever into the chamber. Inactive lever presses had no programmed consequences. Following FR-1 20 s timeout training, we trained rats on a variable-interval 30 s (VI-30) schedule of reinforcement for six 2 h sessions. During the VI-30 sessions, alcohol delivery was available after an active lever press at pseudo-random intervals (1–59 s) after the preceding alcohol delivery.

Phase 3: Punishment: Context B. We trained rats, during 2 h sessions, to self-administer alcohol in an alternate context (Context B) under the same VI-30 schedule of reinforcement mentioned above. Active lever presses resulted in the delivery of 0.1 ml of 20% alcohol paired with the 2 s tone-light cue. Only 50% of the reinforced active lever presses resulted in a 0.5 s footshock (0.1–0.7 mA). Punished active lever presses resulted in

footshock, 2 s tone-light, and alcohol delivery. Inactive lever presses had no reinforced consequences. All rats were punished in Context B for up to 7 d, and footshock intensity was increased by 0.1 mA per session, starting at 0.1 mA. For the CTb+Fos experiment, we increased the shock intensity to 0.7 mA for all rats. For the vSub inactivation and retro-DREADD experiments, we increased the shock intensity to 0.5 mA for all rats and then gave the rats additional punishment sessions at 0.5 mA or 0.6 mA. We then tested the rats after punishment in Context B.

Phase 4: Context-induced relapse (renewal) test. We tested rats for alcohol seeking (active lever presses under extinction conditions) in 90 min (Experiments 1, 2) or 30 min (Experiments 3, 4) sessions in either Contexts A or B (Experiment 1) or in both Contexts A and B (Experiments 2–4). The session duration was only 30 min in Experiments 3 and 4 because of the short half-life of SalB (Vardy et al., 2015). The order of testing in the two contexts was counterbalanced. During the tests, an active lever press, under a VI-30 schedule of reinforcement, resulted in the delivery of the 2 s tone-light cue and the activation of the infusion pump; no alcohol or footshock was delivered.

Surgery

We performed all surgeries after the home-cage alcohol phase of the behavioral procedures. We anesthetized rats with 100 mg/kg ketamine + 10 mg/kg xylazine (i.p.) or isoflurane (5% induction; 2%–3% maintenance) before placing them in the stereotactic frame (Kopf Instruments). We injected the rats with buprenorphine (0.1 mg/kg, s.c.) or ketoprofen (2.5 mg/kg, s.c., Butler Schein) after surgery and the following day (ketoprofen) to relieve pain and decrease inflammation, and gave them 3–5 d to recover after surgery before starting the self-administration sessions.

CTb into NAc shell. We unilaterally injected 40 nl of 1% CTb (List Biological Laboratories) into the NAc shell over 4 min with the needle left in place for an additional 2 min. We gave the injections into either the left or right hemisphere (counterbalanced) using a 1.0 μl, 32 gauge "Neuros" syringe (Hamilton) attached to UltraMicroPump (UMP3) with SYS-Micro4 Controller (World Precision Instruments). The coordinates for NAc shell were as follows: anteroposterior 1.6, mediolateral ±2.3 (10° angle), dorsoventral −7.5 mm from bregma and were based on previous studies (Bossert et al., 2007).

Intracranial cannula surgery (vSub). We implanted bilateral guide cannulas (23-gauge, Plastics One) 1 mm above the vSub. The coordinates for the vSub were as follows: anteroposterior −6.0, mediolateral 5.3 (4° angle), dorsoventral −7.8 mm from bregma (Bossert and Stern, 2014; Bossert et al., 2015). We anchored the cannulas to the skull with jeweler's screws and dental cement.

Viral injections. We bilaterally injected AAV5.CMV.III.GFP-Cre.WPRE.SV40 (UPenn Vector Core lot #V4503MI-R, titer: 9.9×10^{12} gc/ml or V4807MI-3CS, titer: 4.9×10^{12} gc/ml) into NAc shell: anteroposterior 1.6, mediolateral 2.3 (10° angle), dorsoventral −7.5 mm from bregma. We injected 0.75 μl over 3 min and left the needle in place for 2 min. During the same surgery, we bilaterally injected either AAV1.Syn1.dF.HA.KORD.IRES.mCitrine (NIDA Optogenetics and Transgenic Core lot #AAV-2015-03-31-A, titer: 3.0×10^{12} gc/ml) or AAV5.hSyn.DIO.rM3D(GS)-mCherry (UNC Vector Core lot #AV4659c, titer: 6.4×10^{12} gc/ml or AV4659b, titer 4.9×10^{12} gc/ml) into vSub: anteroposterior −6.0, mediolateral 5.3 (4° angle), dorsoventral −8.8 mm from bregma. We injected 1.0 μl over 4 min and left the needle in place for 1 min. Injections were made using 10 μl Nanofil syringes (World Precision Instruments), with 33 gauge needles, attached to a UltraMicroPump (UMP3) with SYS-Micro4 Controller (World Precision Instruments).

Immunohistochemistry

CTb+Fos experiment. Immediately following the behavioral test, we deeply anesthetized rats with isoflurane and transcardially perfused them with ~100 ml of 0.1 M PBS followed by ~400 ml of 4% PFA in 0.1 M sodium phosphate, pH 7.4. We removed brains and postfixed them in 4% PFA for 2 h, then transferred them to 30% sucrose in 0.1 M sodium phosphate, pH 7.4, for 48 h at 4°C. We froze brains on dry ice and stored them at −80°C until sectioning. We cut serial (40 μm) coronal sections using a Leica Microsystems cryostat and stored sections in 0.1 M sodium phosphate, pH 7.4, containing 1% sodium azide at 4°C.

CTb injection site verification. Immunohistochemical procedures were based on our previously published work (Marchant et al., 2009, 2014). We selected a 1-in-4 series of sections from the NAc shell of each rat, and immunofluorescence was used to determine CTb injection sites. We repeatedly rinsed free-floating sections in PBS (3 × 10 min washes) and incubated them for 2 h in PBS containing 0.5% Triton X-100 (PBS-tx) with 10% normal horse serum (NHS). We then incubated all sections for at least 48 h at 4°C in goat anti-CTb primary antibody (1:5000, List Biological Laboratories, 703), and mouse-anti neuronal nuclei primary antibody (1:2000, Millipore, MAB377) diluted in PBS-tx with 2% NHS. We washed off unbound primary antibodies with PBS and incubated the sections for 2–4 h in PBS-tx with 2% NHS and donkey anti-goat AlexaFluor-488 (1:2000; Jackson ImmunoResearch Laboratories, 705-545-147) and donkey anti-mouse AlexaFluor-594 (1:2000; Jackson ImmunoResearch Laboratories, 715-585-151). We then rinsed sections in PBS, mounted onto gelatin-coated glass slides, air-dried, and coverslipped with Mowiol (Millipore).

Fos protein and CTb double labeling. We processed a 1-in-4 series of vmPFC, PVT, BLA, and vSub for immunohistochemical detection of Fos protein and CTb. We rinsed free-floating sections for 30 min in PBS, followed by two 30 min washes in 50% ethanol with the second wash containing 3% hydrogen peroxide and then incubated the sections for 30 min in a blocking solution containing 5% NHS in PBS. We then incubated sections for at least 48 h at 4°C in PBS-tx with 2% NHS, rabbit anti-c-Fos primary antibody (1:8000, Cell Signaling Technology, Phospho-c-Fos, 5348S) and goat anti-CTb primary antibody (1:5000, List Biological Laboratories, 703). Following primary incubation, we rinsed sections in PBS and incubated for 2 h in biotinylated donkey anti-rabbit IgG (1:2000, Jackson ImmunoResearch Laboratories, 711-065-152) diluted in 2% NHS PBS-tx. We rinsed off the secondary antibody with PBS and incubated the sections for 1 h in ABC reagent (Vector Laboratories). Following this step, we incubated sections in 0.1 M sodium acetate with 0.025% DAB in 2% nickel sulfate containing 2 mg/ml D-glucose and 0.4 mg/ml ammonium chloride for 10 min before adding glucose oxidase (0.2 μl per milliliter of solution) to visualize the Fos protein. We stopped the reaction after 10 min with sodium acetate washes. We then rinsed sections in PBS and processed them again in a similar manner, using biotinylated donkey anti-goat IgG (1:2000, Jackson ImmunoResearch Laboratories, 705-065-003) as the secondary antibody for CTb. The DAB step of this reaction occurred without nickel sulfate to visualize CTb as a brown reaction product (6.5 min reaction time). We next mounted the slides onto gelatin-coated glass slides. Finally, we dehydrated the slides through a graded series of alcohol concentrations (30%, 60%, 90%, 95%, 100%, 100% ethanol), cleared with Citrasolv (Fisher Scientific), and coverslipped them with Permount (Fisher Scientific).

DREADD experiments. Following the behavioral testing, we perfused the rats as above and processed the tissue for HA immunoreactivity. We selected a 1-in-4 series of sections from NAc shell and vSub of each rat and used immunofluorescence to determine the total number of KORD (HA-positive) neurons in vSub. We repeatedly rinsed free-floating sections in PBS (3 × 10 min washes) and incubated for 2 h in PBS containing PBS-tx with 10% NHS. We then incubated all sections for at least 48 h at 4°C in rabbit anti-HA primary antibody (1:1000, Cell Signaling Technology, C29F4) diluted in PBS-tx with 2% NHS. We washed off the unbound primary antibodies with PBS and then incubated the sections for 2–4 h in PBS-tx with 2% NHS and donkey anti-rabbit AlexaFluor-594 (1:2000; Jackson ImmunoResearch Laboratories, 711-585-152). We then rinsed sections in PBS, and stained with DAPI (1:1000) for 5 min before mounting onto gelatin-coated glass slides, air-dried, and coverslipped with Mowiol (Millipore).

Image acquisition and neuronal quantification

For CTb+Fos, we digitally captured bright-field images of immunoreactive (IR) cells in the different brain areas using an EXi Aqua camera (QImaging) attached to a Zeiss Axio Imager M2, with the 10× objective. We captured and analyzed the images using iVision (Biovision). Each analyzed image was derived from 5 images captured at different focal planes and then digitally merged using iVision, giving a single image of

in-focus cells. For each rat, we quantified cells in the same hemisphere as the CTb injection. We quantified images of vmPFC (3 sections: bregma 3.72 mm to 2.76 mm), PVT (6 sections: bregma −1.32 mm to −3.48 mm), BLA (4 sections: bregma −2.16 to −3.48 mm), and vSub (2 sections: bregma −5.76 mm to −6.0 mm), by first calculating the area size by outlining the nucleus borders and then manually counting CTb-IR, Fos-IR, and CTb+Fos-IR cells. We present our data as total counts per square millimeter to permit meaningful comparisons between brain regions of different sizes. The counting was performed by an observer that was blind to the experimental conditions.

For HA (KORD), we digitally captured dark-field images of IR cells in vSub using the same microscope with the 5× objective. For each rat, we manually quantified IR cells over 5 sections of vSub (bregma −5.4 mm to −6.2 mm) in both hemispheres. Counting was performed by an observer that was blind to the experimental conditions. For the DREADD experiments, we excluded rats in the SalB condition that had low KORD ($n = 3$) or hM3D ($n = 3$) expression (<20 HA-positive neurons per square millimeter in one or both hemispheres).

Ex vivo *electrophysiology*

We used P rats ($n = 8$) that had undergone 5 d of home-cage access to 20% alcohol and injected bilateral AAV5-Cre in NAc shell and AAV1-DIO-KORD in vSub (as described above). We performed *ex vivo* electrophysiology 6–7 weeks after the surgery. On the recording days, we deeply anesthetized the rats with isoflurane (90–120 s) and then transcardially perfused them with ice-cold solution containing the following (in mM): 220 sucrose, 28 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 7 glucose, 3 sodium pyruvate, and 1 ascorbic acid, saturated with 95% O_2 and 5% CO_2 (Harnett et al., 2012). We cut transverse slices containing vSub in the ice-cold solution and incubated the slices 30 min at 35°C in the same solution (Dougherty et al., 2012). Slices were allowed to recover for a minimum of 1 h at room temperature in ACSF containing the following (in mM): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 21.4 $NaHCO_3$, 11.1 glucose, 3 Na-pyruvate, 1 Na-ascorbate. We recorded from the slices while they were bath perfused (2.5 ml/min) at 32°C–35°C in the same ACSF solution. The intracellular solution contained the following (in mM): 115 K-gluconate, 20 KCl, 1.5 $MgCl_2$, 10 HEPES, 0.025 EGTA, 2 Mg-ATP, 0.2 Na_2-GTP, 10 Na_2-phosphocreatine, pH 7.2–7.3, ~285 mOsm/kg.

We identified KORD-expressing (mCitrine+) cells using scanning disk confocal microscopy (Olympus FV1000) and used differential interference contrast optics to patch the neurons. We performed whole-cell current-clamp recordings in visually identified pyramidal neurons in vSub. We injected current steps during the recording session (500 ms at 0.1 Hz, 50–330 pA). For each cell that we recorded, we generated input–output curves under baseline conditions and following superfusion of vehicle (5% DMSO) for 5–10 min, and finally SalB (100 nM) for 5–10 min (Vardy et al., 2015; Marchant et al., 2016). We excluded recordings if series resistance or input resistance changed >10% during recording session. We used an Axopatch 200B amplifier (Molecular Devices) and Axograph X software (Axograph Scientific) to record and collect the data, which were filtered at 10 kHz and digitized at 4–20 kHz.

Specific experiments

Experiment 1: Effect of context-induced relapse of alcohol seeking after punishment-imposed abstinence on Fos expression in projections to NAc shell (n = 24). We trained rats to self-administer alcohol in Context A and subsequently punished alcohol-reinforced responding in Context B. We gave all rats 7 punishment sessions before the context-induced relapse test, with the shock intensity increasing from 0.1 to 0.7 mA in 0.1 mA increments. Following punishment-imposed suppression of alcohol taking, we tested the rats in Context A (alcohol training context; $n = 8$), Context B (punishment context; $n = 8$), or did not test them (No-test group; $n = 8$). Immediately after the 90 min test, we deeply anesthetized the rats with isoflurane and extracted their brains after perfusion. The No-test rats were taken directly from their home-cage and perfused on the same day as the other rats. We then processed brains for immunohistochemical detection of Fos protein and CTb. The number of rats we used for the Fos+CTb double-labeling measurement was lower than for the Fos measurement because we excluded rats with low CTb expression. Additionally, the number of rats for the Fos assay was not identical for the different brain regions because of loss of tissue during cryostat sectioning or during the immunohistochemistry assay.

Experiment 2: Effect of vSub inactivation on context-induced relapse of alcohol seeking after punishment imposed abstinence (n = 14). We trained rats to self-administer alcohol in Context A and subsequently punished the alcohol-reinforced responding in Context B. We gave all rats 7 punishment sessions before the context-induced relapse test, with the shock intensity increasing from 0.1 mA to 0.5 mA in 0.1 mA increments. On punishment days 6 and 7, we kept the shock intensity at 0.5 mA. We tested all rats in 90 min sessions in both Context A and Context B under extinction conditions over consecutive days (order counterbalanced). The groups were vSub muscimol+baclofen ($n = 7$) and vSub vehicle ($n = 7$).

Intracranial injections. We dissolved muscimol+baclofen (Tocris Bioscience; concentration: 0.06 + 0.6 mM or 3.6 + 64.1 ng in 0.5 μl per side) in sterile saline. The injectors extended 1 mm below the tips of the guide cannulas. We injected vehicle (0.9% saline) or muscimol+baclofen over 1 min and left the injectors in place for 1 min; we tested the rats 5–10 min after injections. We used a Harvard Apparatus syringe pump connected to 10 μl Hamilton syringes attached via polyethylene-50 tubing to 30 gauge injectors for all intracranial injections. Following the final test, we deeply anesthetized rats with isoflurane and removed their brains and stored them in 10% formalin. We sectioned brains at 40 μm using a Leica Microsystems cryostat and stained sections with cresyl violet to verify the placement of the cannulas.

Experiment 3: Effect of SalB injections on context-induced relapse in rats expressing KORD in vSub→NAc shell neurons (n = 29). We injected the rats with AAV5-Cre into NAc shell and AAV1-DIO-KORD into vSub after the home-cage phase. We then trained the rats to self-administer alcohol in Context A and subsequently punished the alcohol-reinforced responding in Context B. We habituated the rats to the DMSO injections by giving them 0.2 ml (s.c.) injections 10 min before 3 sessions in training Context A and 1 session in punishment Context B. We gave all rats 6 punishment sessions, with the shock intensity increasing from 0.1 mA to 0.5 mA in 0.1 mA increments. On punishment day 6, all rats that had >15 active lever presses on punishment day 5 received 0.6 mA; the rest of the rats received 0.5 mA on punishment day 6. For the relapse tests, we used an experimental design that included the within-subjects factors of test context (Context A, Context B) and the between-subjects factor of SalB dose (vehicle [DMSO], 15 mg/kg, s.c; injection volume 0.5 ml/kg; 10 min pretreatment time). We tested the rats for context-induced relapse under extinction conditions in Contexts A and B (30 min duration) over 2 d. We counterbalanced the order of testing in Contexts A and B. After these tests, we gave the rats an additional Context B punishment session (0.5 mA). We then retrained them to self-administer alcohol in Context A (3 daily sessions), repunished them in Context B (3 sessions; 0.3, 0.4, 0.5 mA), and retested them under extinction conditions in Contexts A and B with the drug condition reversed. We added a retest component to Experiment 3 because in unpublished studies we observed a success rate of ~60% for high HA expression in both hemispheres. Thus, we were hoping that, with repeated testing, we will have a sufficient number of subjects for a complete within-subjects assessment of the effect of SalB versus vehicle on context-induced relapse after punishment. We ended up not using this experimental approach because initial inspection of the data showed both an order effect (higher active lever presses during the second test than during the first test) and an unexpected dissociation of the effect of SalB on the first versus the second test (see Results and Discussion).

Experiment 4: Effect of SalB injections on context-induced relapse in rats expressing hM3D in vSub→NAc shell neurons (n = 24). The goal of this control experiment was to determine whether SalB injections will have an effect on context-induced relapse in rats injected with AAV5-Cre in NAc shell and a different DREADD (AAV5-DIO-hM3D) in vSub that is not activated by SalB. The procedures and experimental design were identical to those described for Experiment 3, except that we injected the rats with AAV5-DIO-hM3D into the vSub after the home-cage phase and only tested them once. We chose to use AAV5-DIO-hM3D as a control virus

instead of typical fluorescent protein controls (e.g., AAV5-DIO-YFP, AAV5-DIO-GFP) because this viral construct, like AAV1-DIO-KORD, expresses a membrane-targeted, ligand-activated designer receptor that has no effect on basal neuronal activity in the absence of the ligand.

Statistical analysis

We analyzed the data separately for the four phases: (1) home-cage alcohol intake; (2) Context A training; (3) Context B punishment; and (4) context-induced relapse tests. For the relapse tests, the dependent variable was the total number of active lever presses or number of minutes to first active lever press (latency); the number of inactive lever presses during testing was used as a covariate in the analyses. We analyzed the immunohistochemical data with cell counts per square millimeter of a given brain region as the dependent variable. In Experiment 1, we analyzed the number of Fos-IR, CTb-IR, and CTb+Fos cells using a mixed ANOVA with the within-subjects factor of brain region and the between-subjects factor of test context (Context A, Context B, No-test). We subsequently analyzed main effects using one-way ANOVAs. In Experiment 2 (vSub inactivation), we analyzed the relapse test data using the within-subjects factor of test context (Context A, Context B) and the between-subjects factor of drug (vehicle, muscimol+baclofen). In Experiments 3 and 4, we analyzed the relapse test data using the within-subjects factors of test context (Context A, Context B) and the between-subjects factor of SalB (vehicle, 15 mg/kg). In the *ex vivo* electrophysiology experiments, we analyzed the number of action potentials using the within-subjects factors of current (50–330 pA in 20 pA steps) and SalB concentration (0, 100 nM) and the between-subjects factor of KORD expression (KORD-positive or KORD-negative).

We performed all statistical analyses using IBM SPSS version 21 and followed up on significant main or interaction effects ($p < 0.05$) with Fisher PLSD *post hoc* tests. We present the data in the figures as mean ± SEM.

Results

Training and punishment phases

The alcohol-preferring P rats consumed high amounts of alcohol in the home-cage intermittent-access choice phase and reliably self-administered alcohol in Context A under the FR-1, 20 s timeout and VI-30 schedules of reinforcement (Fig. 1). During punishment in Context B, the rats decreased alcohol self-administration when we increased the shock intensity (Fig. 1).

Effect of context-induced relapse after punishment-imposed abstinence on neuronal activity in neurons that project to NAc shell

In Experiment 1, we combined retrograde tracing via CTb (injected into NAc shell), with measurement of Fos in the projection areas, to determine whether context-induced relapse to alcohol seeking after punishment-imposed abstinence is associated with activation of vSub, vmPFC, PVT, and BLA neurons projecting to NAc shell. Below, we first present the behavioral data and then describe the neuroanatomical data.

Context-induced relapse test (behavioral data)

We observed context-induced relapse to alcohol seeking in Context A after punishment-imposed abstinence in Context B (Fig. 2*C*). An ANCOVA of active lever presses (inactive lever presses as covariate) showed a significant effect of test context ($F_{(1,13)} = 40.7, p < 0.01$). An analysis of the latency (time to first active lever press) data showed a significant effect of test context ($F_{(1,14)} = 14.0, p < 0.01$); Figure 2*D*).

Fos data

Our initial analysis using the within-subjects factor brain region (vSub, BLA, PVT, and vmPFC) and the between-subjects factor test context showed a significant interaction between the two factors ($F_{(6,54)} = 3.8, p < 0.01$). Subsequent one-way ANOVAs within each brain region showed a significant effect of test context in each brain region (vSub: $F_{(2,18)} = 9.6$; BLA: $F_{(2,19)} = 6.2$; PVT: $F_{(2,19)} = 10.5$; and vmPFC: $F_{(2,19)} = 6.9$; p values <0.01). *Post hoc* analyses showed significant differences between Context A group compared with Context B and No-test groups for vSub, BLA, and PVT (p values <0.05; Fig. 2*F*). In the vmPFC, the number of Fos-IR in Context A group was significantly different from the No-test group ($p < 0.01$) but not Context B group ($p = 0.09$; Fig. 2*F*).

CTb+Fos data

There were no differences observed in the total number of CTb-IR cells across groups (p values >0.1; Fig. 2*G*). Analysis of the CTb+Fos-IR counts showed a significant interaction between brain region (within-subjects factor) and test context (between-subjects factor) ($F_{(4,30)} = 11.4, p < 0.01$). Subsequent one-way ANOVAs showed a significant effect of test context on the number of CTb+Fos-IR cells in vSub ($F_{(2,16)} = 23.7, p < 0.01$) but not BLA, PVT, or vmPFC (p values >0.1). *Post hoc* analyses of the vSub counts showed higher CTb+Fos-IR cells in rats tested in Context A compared with rats tested in Context B or the No-test group (p values <0.01; Fig. 2*H*).

In summary, the data in Experiment 1 confirm our previous results that exposure to the original alcohol-associated training context, after punishment-imposed abstinence in a different context, provokes relapse to alcohol seeking (Marchant et al., 2013). More importantly, the CTb+Fos double-labeling data show that context-induced relapse after punishment-imposed abstinence is associated with selective activation of vSub neurons that project to NAc shell.

Effect of vSub inactivation on context-induced relapse to alcohol seeking after punishment-imposed abstinence

In Experiment 2, we determined the causal role of vSub in context-induced relapse by reversible inactivation of vSub, using intracranial injections of GABAa+GABAb receptor agonists (muscimol+baclofen) (McFarland and Kalivas, 2001), before the context-induced relapse test.

We found that vSub inactivation decreased context-induced relapse to alcohol seeking after punishment-imposed abstinence (Fig. 3*B*). Analysis of active lever presses (inactive lever presses as a covariate) showed a significant interaction between test context (Context A, Context B; within-subjects) and drug (muscimol+baclofen, vehicle; between-subjects) ($F_{(1,10)} = 7.8, p < 0.05$). Analysis of latency to first active lever press revealed a significant main effect of test context ($F_{(1,12)} = 9.0, p < 0.05$) but no significant interaction between drug and test context ($p > 0.05$; Fig. 3*B*). These data indicate that vSub inactivation decreases alcohol seeking in Context A without affecting the latency to initiate alcohol seeking during the relapse tests.

The results of Experiment 2, combined with the results of Experiment 1, indicate that neuronal activity in vSub plays a critical role in context-induced relapse after punishment-imposed abstinence.

Ex vivo electrophysiology validation of the inhibitory action of SalB in KORD expressing vSub→NAc shell neurons

We used *ex vivo* electrophysiology to verify that our double-virus "retro-DREADD" procedure (Marchant et al., 2016) permits DREADD-mediated selective inhibition of vSub→NAc shell neurons. We found that bath application of SalB decreased spike rate induced by direct current injection in KORD-positive neurons (identified by expression of mCitrine) but not KORD-negative neurons. The statistical analysis consisted of the

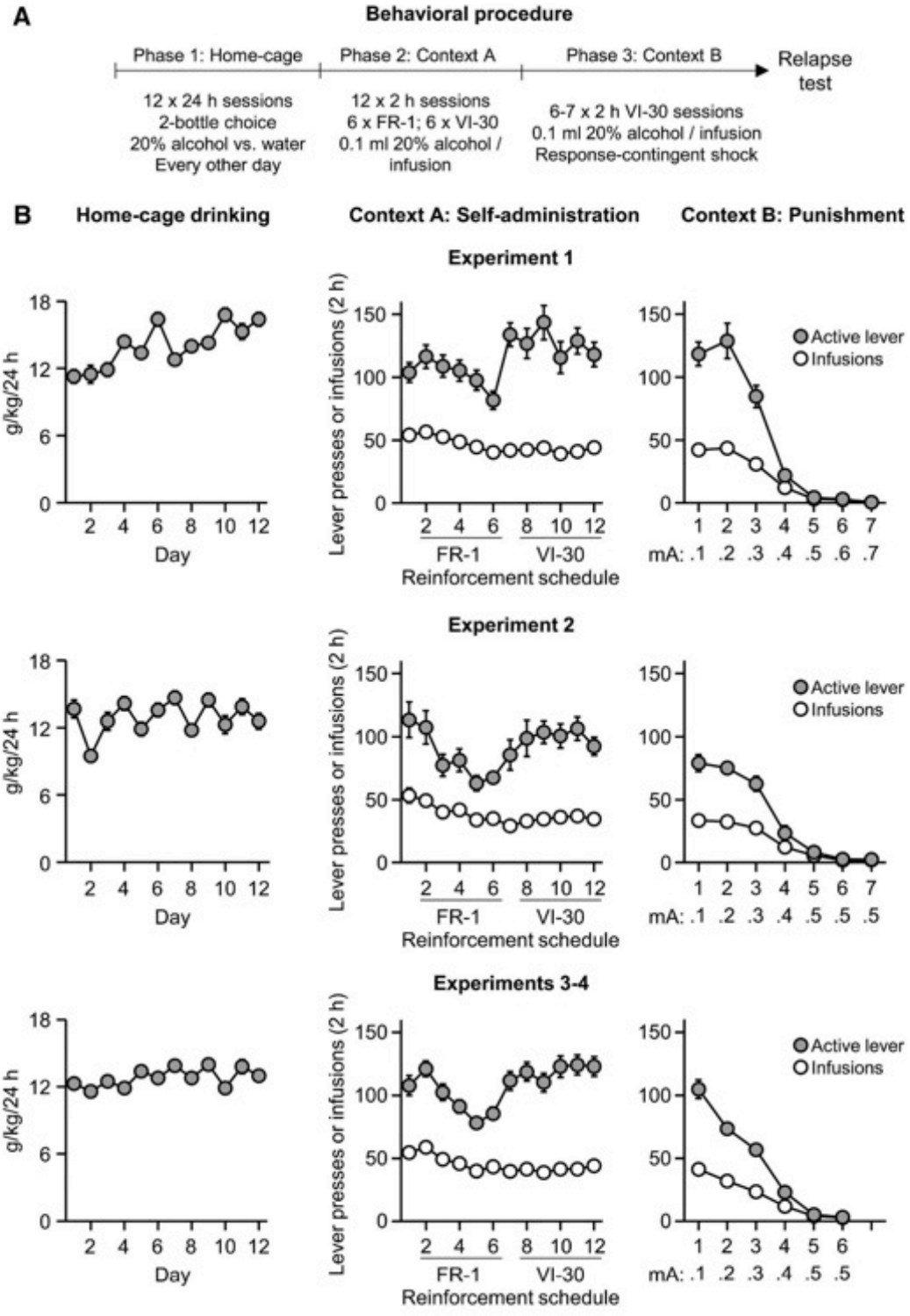

Figure 1. Home-cage intake, alcohol self-administration in Context A, and punishment of alcohol self-administration in Context B. ***A***, Outline of the experimental procedure before the context-induced relapse tests. FR, Fixed-ratio. ***B***, Alcohol intake (grams per kilogram) during home-cage access to 20% alcohol. Context A active lever presses and infusions under the FR-1 20 s timeout and VI-30 schedules of reinforcement. Context B active lever presses and infusions. Data are mean ± SEM; $n = 91$. Experiment 1 shock punishment range, 0.1–0.7 mA, $n = 24$. Experiment 2 shock punishment range, 0.1–0.5 mA, $n = 14$. Experiments 3 and 4 shock punishment range, 0.1–0.5 mA, $n = 53$.

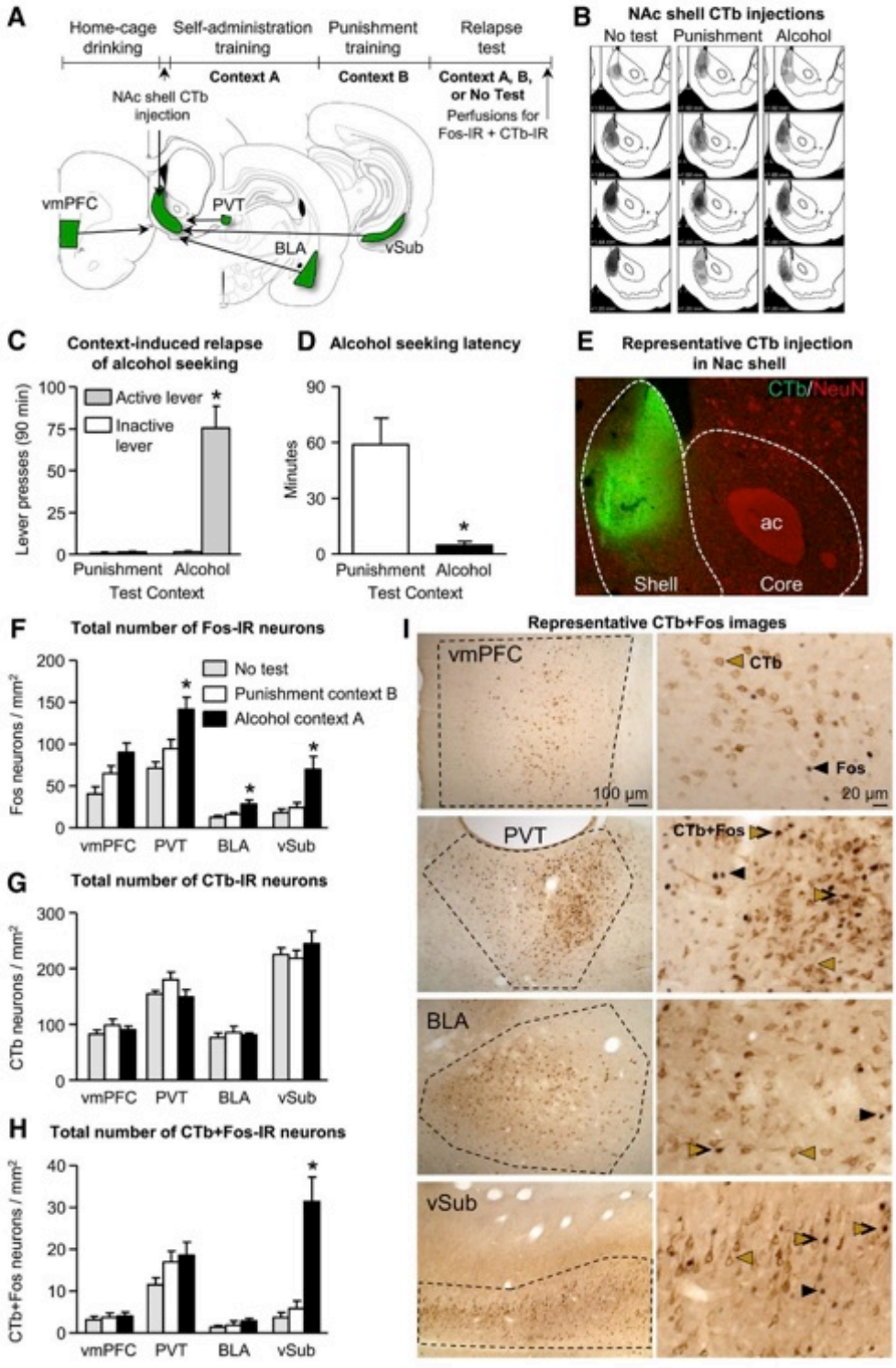

Figure 2. Context-induced relapse after punishment-imposed abstinence is associated with selective activation of the vSub→NAc projection. ***A***, Outline of experimental procedure and schematic of the inputs to NAc shell assessed in Experiment 1. ***B***, Representative plots of the spread of CTb injections for the three groups. ***C***, Data are mean ± SEM; number of active and inactive lever presses during the relapse test. ***D***, Data are mean ± SEM; latency to first active lever press during the relapse test. ***E***, Photomicrograph of CTb injection contained to the NAc shell (green) with background staining of neuronal nuclei (NeuN). ***F***, Data are mean ± SEM; number of Fos-IR neurons per square millimeter in vmPFC, PVT, BLA, and vSub in rats tested in either Context A, Context B, or not tested. ***G***, Data are mean ± SEM; number of CTb-IR neurons per square millimeter in vmPFC, PVT, BLA, and vSub. ***H***, Data are mean ± SEM; number of CTb + Fos-IR neurons per square millimeter in vmPFC, PVT, BLA, and vSub. *$p < 0.05$. $n = 5–8$ per group. ***I***, Representative photomicrographs of CTb and Fos-IR in each brain region analyzed. *Different from the Punishment condition (***C***, ***D***) or from the Punishment and No-test conditions (***F***, ***H***), $p < 0.05$.

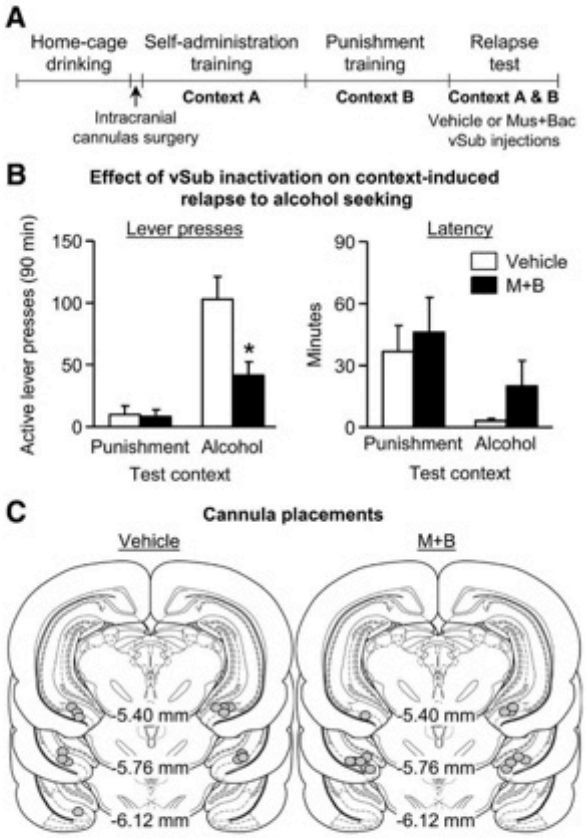

Figure 3. vSub inactivation decreases context-induced relapse to of alcohol seeking after punishment-imposed abstinence. ***A***, Outline of experimental procedure. ***B***, Data are mean ± SEM; number of active lever presses (left) and active lever latency (right) during the relapse test. $n = 7$ per group. ***C***, Approximate cannula placements (mm from bregma) of the injector tips for vSub. M, muscimol; B, baclofen. *Different from the Vehicle condition, $p < 0.05$.

between-subjects factor of KORD expression (KORD-positive, $n = 8$ cells; KORD-negative, $n = 7$ cells; from 8 rats in total), and the within-subjects factors current (50–330 pA in 20 pA steps) and SalB concentration (0, 100 nM). The statistical analysis showed a significant current X SalB concentration × expression interaction ($F_{(28,364)} = 4.7$, $p < 0.01$; Fig. 4*D*,*E*). The results of our electrophysiology study show selective inhibition of neuronal firing by SalB in KORD-positive vSub→NAc shell neurons, but not KORD-negative neurons, setting the stage for Experiment 3 (described below) on the effect of inhibition of the vSub→NAc shell projection on context-induced relapse after punishment-imposed abstinence.

Effect of chemogenetic inhibition of vSub→NAc shell projection neurons on context-induced relapse of alcohol seeking after punishment-imposed abstinence

In Experiment 3, we used the "retro-DREADD" dual-virus approach (Marchant et al., 2016) to restrict expression of KORD in vSub to neurons projecting to NAc shell (Fig. 5*B*) to determine the causal role of the vSub→NAc shell projection in context-induced relapse after punishment-imposed abstinence. Based on the results of Experiments 1 and 2, we predicted that systemic injections of the designer drug SalB before the relapse test, which will selectively inhibit neuronal activity in the vSub→NAc shell projection during the test, would decrease context-induced relapse.

The mean ± SEM number of HA-positive neurons per hemisphere in the vehicle ($n = 16$) and SalB ($n = 13$) group was 27.5 ± 4.3 and 49.8 ± 4.4, respectively. We eliminated 3 rats from the SalB group because of low KORD expression (the mean ± SEM number of HA-positive neurons in the lowest hemisphere of these rats was 7.0 ± 3.2). We did not exclude any rats from the vehicle group to maintain sufficient statistical power and because there was no difference in alcohol self-administration in Context A or punishment responding in Context B between rats that were injected with vehicle during the first context-induced relapse test versus the high KORD-expressing rats that were injected with SalB during this test (data not shown).

We found that SalB decreased context-induced relapse on the first relapse test (Fig. 5*C*). The analysis of active lever presses of this test included the between-subjects factor of SalB dose (vehicle, 15 mg/kg) and the within-subjects factors of test context (Context A, Context B) with inactive lever presses as the covariate. This analysis showed significant main effects of test context ($F_{(1,25)} = 47.5$, $p < 0.01$) and SalB dose ($F_{(1,25)} = 5.6$, $p < 0.05$), and a significant interaction between the two factors ($F_{(1,25)} = 4.9$, $p < 0.05$). The statistical analysis of the latency for the first response showed a significant main effect of test context ($F_{(1,27)} = 34.6$, $p < 0.01$) but no significant main effect of SalB dose or interaction between the two factors (Fig. 5*C*). These data show that, during the first relapse test, inhibition of the vSub→NAc shell projection decreased context-induced relapse to alcohol seeking but had no effect on the latency to the first response.

As described in Materials and Methods, at the end of Experiment 3, we retrained, repunished, and retested the rats for context-induced relapse. The retest conditions were the same as in the initial test, except that the rats injected with vehicle during the first test, were injected with SalB during the second test and vice versa. The retest condition included all rats injected with SalB on Test 1 and then injected with vehicle on Test 2 ($n = 13$) and a subgroup of rats ($n = 6$) injected with vehicle on Test 1 and SalB on Test 2 that were equated for vSub KORD expression to the first group (45.4 ± 4.6 vs 49.8 ± 4.4 HA-positive neurons per square millimeter). Unexpectedly, after additional exposure to alcohol and punishment, inhibition of the vSub→NAc shell projection with SalB had no effect on context-induced relapse. The mean ± SEM number of active lever presses per 30 min in Contexts A and B after vehicle ($n = 13$) or SalB ($n = 6$) injections was 62.2 ± 5.2 and 65.8 ± 14.8, and the number of inactive lever presses was 1.0 ± 0.5 and 6.2 ± 4.1. The statistical analysis showed a significant effect of test context ($F_{(1,15)} = 62.0$, $p < 0.01$) but no significant main effect of SalB dose or interaction between the two factors. Importantly, reanalysis of the Test 1 data in the same subsample of rats analyzed in Test 2 (i.e., equated for vSub KORD expression), SalB injections decreased context-induced relapse during the first test: mean ± SEM of 58.5 ± 9.6 (vehicle) and 30.9 ± 6.5 (SalB) lever presses per 30 min ($F_{(1,15)} = 5.7$, $p < 0.05$).

Finally, in Experiment 4, we determined the effect of SalB injections on context-induced relapse in rats injected with AAV5-Cre into NAc shell and with AAV5-DIO-hM3D into vSub, a different DREADD that is not activated by SalB (Armbruster et al., 2007). The procedures and experimental design of this control experiment were identical to those of the first test in Experiment 3. Three rats were eliminated from the SalB condition because of missing vSub sections or low expression. The mean ± SEM number of lower and higher hemisphere hM3D-expressing vSub neurons per square millimeter in the SalB condition was 75.8 ± 10.7 and 98.2 ± 10.5, respectively.

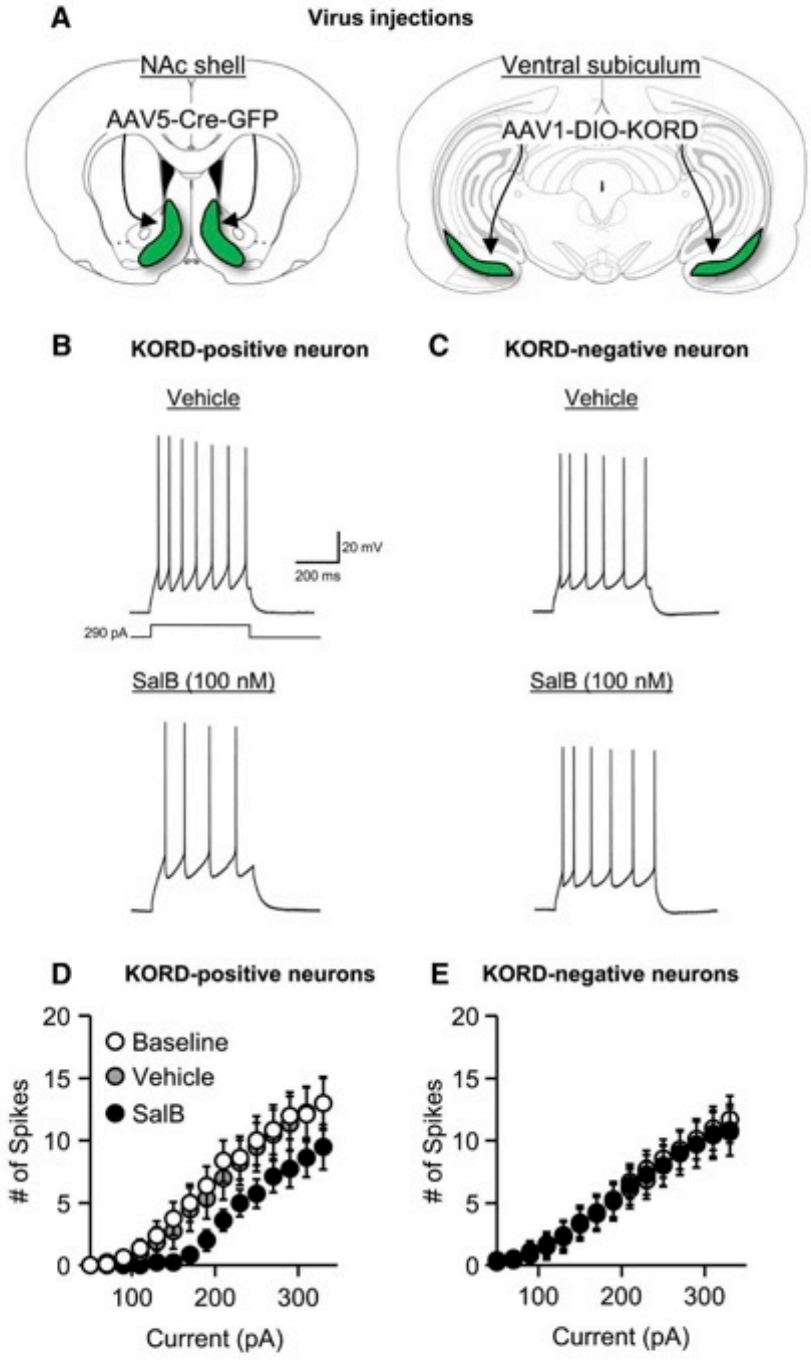

Figure 4. SalB inhibits neural activity in KORD-expressing vSub→NAc shell neurons. ***A***, Schematic of AAV5-Cre injections in NAc shell and AAV1-DIO-KORD injections in vSub. ***B***, Example trace of action potentials evoked by current injection in KORD-positive neuron in the vehicle condition (top) and SalB condition (bottom). ***C***, Example trace of action potentials evoked by current injection in a KORD-negative neuron in the vehicle condition (top) and SalB condition (bottom). ***D***, Group data for firing frequency plotted as a function of current injection in KORD-positive vSub→NAc shell neurons; $n = 8$. ***E***, Group data for firing frequency plotted as a function of current injection in KORD-negative neurons; $n = 7$.

In rats with vSub expression of hM3D, we found that SalB injections had no effect on context-induced relapse after punishment-imposed abstinence. The mean ± SEM number of active lever presses in Context A were as follows: vehicle ($n = 11$), 29.5 ± 5.6; and SalB ($n = 13$), 34.1 ± 7.0. The mean ± SEM number of active lever presses in Context B was as follows: vehicle 0.45 ± 0.25; SalB 0.46 ± 0.18. The statistical analysis showed a significant effect of test context ($F_{(1,20)} = 25.2$, $p < 0.01$) but no other significant main or interaction effects. The statistical analysis on the latency for the first response showed a significant main effect of test context ($F_{(1,22)} = 60.2$, $p < 0.01$) but no significant main effect of SalB dose or interaction between the two factors. These data show that SalB injections had no effect on context-induced relapse in rats expressing hM3D in vSub→NAc shell neurons.

Together, the results of Experiments 3 and 4 suggest a time-limited role of the vSub→NAc shell projection in context-induced relapse after punishment-imposed abstinence (see Discussion).

Discussion

We report four main findings. First, context-induced relapse after punishment-imposed abstinence was associated with selective activation of the vSub→NAc shell projection. Second, reversible inactivation of vSub decreased context-induced relapse. Third, chemogenetic inhibition of the vSub→NAc shell projection decreased context-induced relapse, albeit to a lesser degree than vSub reversible inactivation. Fourth, the effect of chemogenetic inhibition of the vSub→NAc shell projection was designer-receptor specific and dependent on prior history of alcohol and punishment exposure. SalB had no effect on context-induced relapse in rats expressing hM3D in the vSub→NAc shell projection or following reacquisition and repunishment in rats expressing KORD in this projection. Our results demonstrate a critical role of vSub in context-induced relapse after punishment-imposed abstinence and suggest that this effect involves the vSub→NAc shell projection during the first relapse episode.

Role of ventral subiculum in context-induced relapse

Our results demonstrate a critical role of vSub in context-induced relapse to alcohol seeking after punishment-imposed abstinence. These findings extend previous results on the role of vSub (Bossert and Stern, 2014) and the vSub→NAc shell projection (Bossert et al., 2015) in context-induced reinstatement of heroin seeking after extinction in which we used a traditional anatomical disconnection procedure (Gold, 1966). There is also evidence for a role of ventral hippocampal areas, located dorsal to vSub, in context-induced reinstatement of cocaine seeking after extinction (Lasseter et al., 2010).

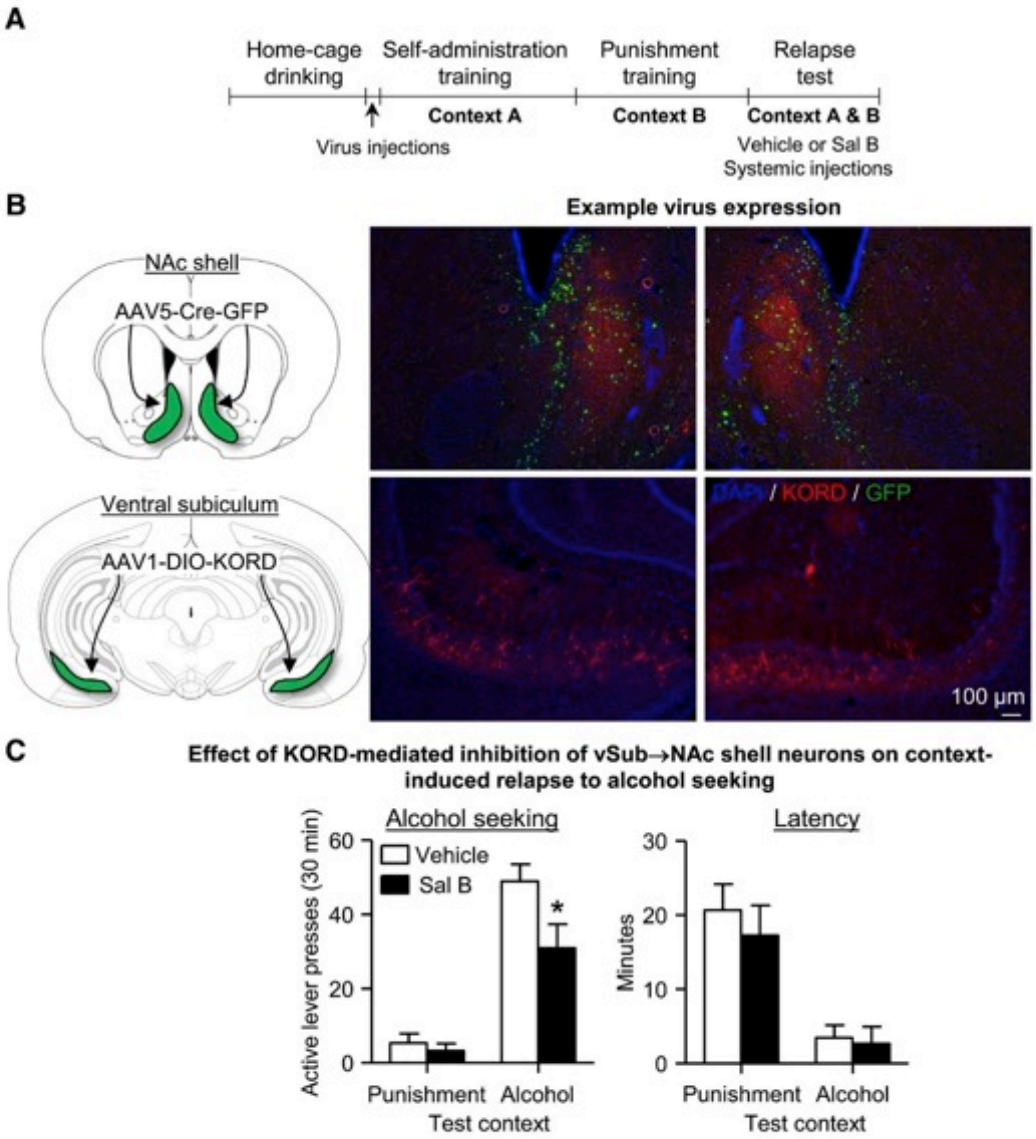

Figure 5. Chemogenetic inhibition of vSub→NAc shell projection decreases context-induced relapse of alcohol seeking after punishment-imposed abstinence. ***A***, Outline of experimental procedure. ***B***, Schematic of AAV5-Cre injections in NAc shell and AAV1-DIO-KORD injections in vSub. ***C***, Data are mean ± SEM; number of active lever presses (left) and latency to first active lever press (right) during the first relapse test. $n = 13–16$ per group. *Different from the Vehicle condition, $p < 0.05$.

Thus, although a role of vSub in context-induced reinstatement of alcohol seeking after extinction has not been established, a tentative conclusion is that vSub and vSub→NAc shell projections are critical for context-induced relapse independent of the self-administered drug and the mechanism used to impose abstinence. Support for this notion comes from the findings that NAc shell is critical for context-induced reinstatement after extinction across drug classes (Bossert et al., 2007; Fuchs et al., 2008b; Chaudhri et al., 2009) and context-induced relapse to alcohol seeking after punishment (Marchant and Kaganovsky, 2015).

The vSub and other subregions of the ventral hippocampus have been implicated in other forms of context-dependent learning (Bannerman et al., 2004). For example, excitotoxic lesion or reversible inactivation of ventral hippocampus decreases renewal of conditioned fear (Maren, 1999; Hobin et al., 2006). Thus, one potential account of the present and previous results is that the primary function of ventral hippocampus is to encode the motivational significance of emotionally relevant contexts. However, there are several studies implicating vSub in drug seeking in experiments where the context was not manipulated. For example, electrical stimulation of vSub reinstates cocaine or amphetamine seeking after extinction (Vorel et al., 2001; Tapavarapruk et al., 2015). Inactivation of ventral hippocampus or vSub with muscimol+baclofen or lidocaine decreases cue and drug priming-induced reinstatement of cocaine and methamphetamine seeking (Sun and Rebec, 2003; Hiranita et al., 2006; Rogers and See, 2007). Additionally, inactivation of ventral hippocampus with muscimol+baclofen decreases the effect of repeated restraint stress, given during abstinence, on enhanced reacquisition of nicotine self-administration (Yu and Sharp, 2015). These data point to a more general role of vSub in promoting relapse to drug seeking.

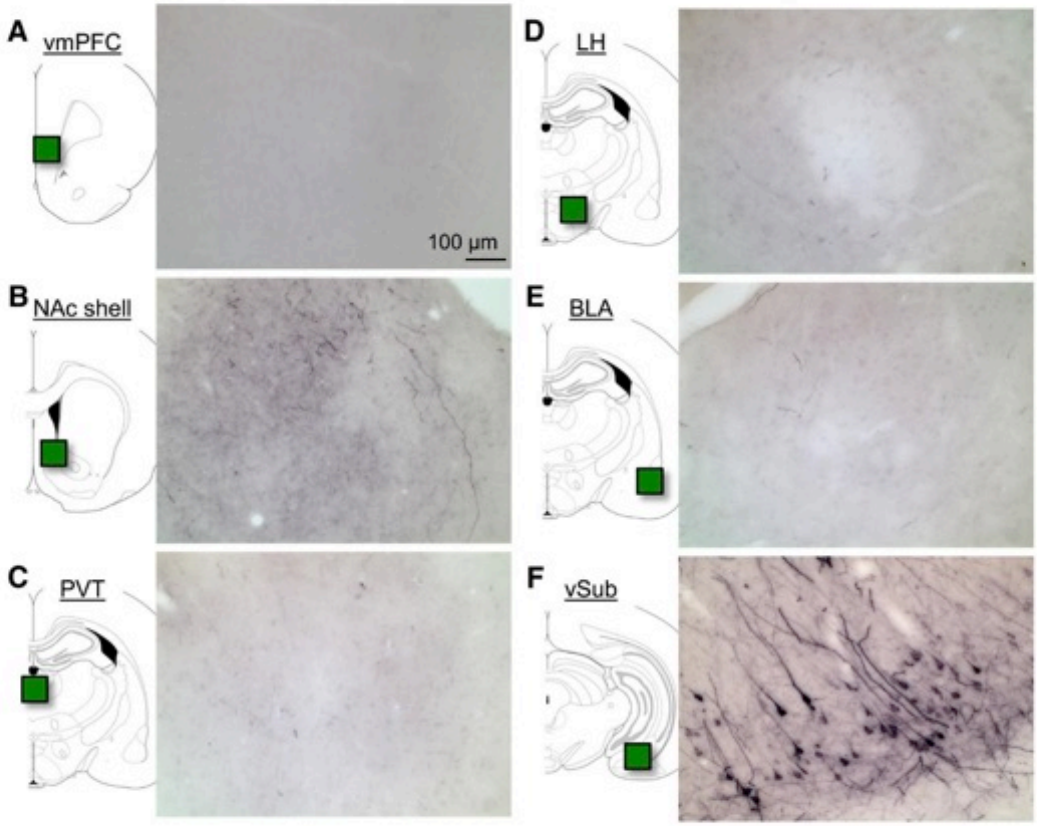

Figure 6. vSub→NAc shell projections do not send collateral projections to other vSub projection areas. Representative photomicrographs of HA immunoreactivity from a rat in Experiment 3 that received AAV5-Cre in NAc shell and AAV1-DIO-KORD in vSub. ***A***, vmPFC. ***B***, NAc shell. ***C***, PVT. ***D***, Lateral hypothalamus (LH). ***E***, BLA. ***F***, vSub.

Finally, anatomical and synaptic physiology studies suggest that vSub→NAc shell projection neurons are glutamatergic (Christie et al., 1987; Britt et al., 2012). There is also evidence that glutamate and dopamine (via D1 receptors) act in synergy to promote activity of NAc neurons (O'Donnell, 2003; Floresco, 2007). Thus, we propose that vSub glutamate release in NAc shell acts in combination with dopamine, originating from the ventral tegmental area (Nauta et al., 1978), to promote context-induced relapse to alcohol seeking. Evidence supporting this idea is that chemical and electrical vSub stimulation increases NAc dopamine release (Legault et al., 2000; Taepavarapruk et al., 2000), an effect that is dependent on both ventral tegmental area activity (Legault et al., 2000) and NAc glutamate receptors (Taepavarapruk et al., 2000; Floresco et al., 2001). Additionally, we recently found that blockade of NAc shell dopamine D1 receptors decreases context-induced relapse to alcohol seeking after punishment-imposed abstinence (Marchant and Kaganovsky, 2015).

Methodological and conceptual considerations

Several issues should be considered in the interpretation of the present data. One issue is whether our findings from experiments using the genetically selected P rats generalize to outbred rat strains that consume less alcohol than the P rats. Another issue is that the effects on context-induced relapse of vSub reversible inactivation or chemogenetic inhibition of vSub→NAc shell projections are due to nonspecific performance deficits. This is unlikely because we previously found no effect of vSub muscimol+baclofen injections on high-rate operant responding for food (Bossert and Stern, 2014), and SalB had no effect on context-induced relapse in rats expressing hM3D in vSub→NAc shell neurons. A second issue is that the lower vSub KORD (HA-IR) expression in the vehicle-injected rats (compared with the SalB-injected rats) contributed to the group differences in context-induced relapse (Fig. 5*C*). This is unlikely because the two groups did not differ in their response latency during testing or in other behavioral measures (acquisition and reacquisition of alcohol self-administration, punishment, and repunishment responding (data not shown).

Another issue is the relative contribution of the vSub→NAc projection to the role of vSub in context-induced relapse after punishment. As seen in Figures 3 and 5, we observed a greater reduction of alcohol seeking after vSub inactivation than after chemogenetic inhibition of vSub→NAc shell projections. A potential interpretation of this difference is that vSub projections to other brain regions also play a role in context-induced relapse. Although this possibility cannot be ruled out, we speculate that the weaker effect of our retro-DREADD manipulation is likely due to the fact that this manipulation only partially inhibited the activity of the projection because not all projection neurons were expressing KORD. We observed approximately half the number of HA-positive neurons in Experiment 3 compared with the number of CTb-positive neurons in Experiment 1. Thus, the observed behavioral effects of SalB may underestimate the contribution of the vSub→NAc shell projection to context-induced relapse. In this regard, the serotype of the KORD virus we used in the present study was different from the one we used in our

previous study (Marchant et al., 2016), resulting in a lower number of KORD-expressing neurons in the present study. Another issue related to the retro-DREADD methodology is that the observed behavioral effects may be due to collateral projections to brain areas other than those injected with the retrograde AAV-Cre. This possibility is unlikely in our study because very few vSub projection neurons to a given brain area send collaterals to other brain areas (Namura et al., 1994; Naber and Witter, 1998). Indeed, as can be seen in Figure 6, using this dual-virus strategy, we observe HA labeling of terminals in NAc shell but not in other vSub projection areas.

Another issue to consider is that our experimental manipulations (vSub inactivation, chemogenetic inhibition of the vSub→NAc shell projection) had no effect on response latency during testing. These results suggest that neuronal activity in vSub and vSub→NAc shell projection does not mediate the initial context-driven alcohol seeking but underlies other learning and motivational processes that control drug seeking during the nonreinforced relapse tests.

Finally, an unexpected finding in our study was that chemogenetic inhibition of the vSub→NAc shell projections had no effect on context-induced relapse after reacquisition and repunishment. The reasons for this time-limited role of vSub→NAc shell projection in context-induced relapse are unknown and may be due to multiple factors, including the rat strain, the effectiveness of neuronal inhibition by KORD, and other procedural factors. Noteworthy, relevant literature does not exist because, over the last decades, studies using relapse models have not compared mechanisms of initial versus repeated relapse/reinstatement episodes after reacquisition of drug self-administration (Venniro et al., 2016). However, for at least one form of relapse, reacquisition after extinction (Millan et al., 2013; McNally, 2014), there is evidence for pharmacological dissociation of reacquisition versus initial acquisition of alcohol self-administration: the former is naloxone-sensitive, whereas the latter is not (Perry and McNally, 2012). Based on these findings, one potential account of our data, which can only be resolved by future studies, is that mechanisms underlying the initial relapse episode might be different from those involved in subsequent relapse episodes.

Concluding remarks

We used our recently developed model of context-induced relapse where abstinence is voluntary and studied the role of projections to NAc shell in this form of relapse. Our results demonstrate a critical role of vSub in context-induced relapse after punishment-imposed abstinence and further suggest a role of the vSub→NAc shell projection in this form of relapse. A surprising finding was that chemogenetic inhibition of vSub→NAc shell projection, which decreased first-episode context-induced relapse, had no effect on relapse after reacquisition of alcohol self-administration and repunishment. This unexpected finding may have implications for future studies on mechanisms of drug relapse in rat models. Over the years, relapse studies in the addiction field have been exclusively limited to the investigation of an initial relapse episode induced by exposure to drug priming, stress, and drug cues or contexts (Kalivas and McFarland, 2003; Bossert et al., 2013; Mantsch et al., 2016; Venniro et al., 2016). Our serendipitous findings on the dissociable effects of SalB injections on the first versus second context-induced relapse episode after reacquisition and repunishment suggest that studies on mechanisms underlying relapse after multiple prior relapse episodes, which commonly occurs in most alcohol and drug users (O'Brien, 1997; Heilig et al., 2011), are warranted. Finally, based on previous studies on the differences in mechanism of drug reward and relapse across drug classes (Badiani et al., 2011; Badiani, 2013), another question for future research is whether the circuit mechanism identified here generalizes to other drugs of abuse.

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CHAPTER THREE

Chemogenetic activation of the lateral hypothalamus reverses early life stress-induced deficits in motivational drive

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Jiann Wei Yeoh	Analyzed data and wrote the manuscript	
Deborah M. Hodgson	Wrote the manuscript	
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Christopher V. Dayas	Designed research, analyzed data and wrote the manuscript	

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CHEMOGENETIC ACTIVATION OF THE LATERAL HYPOTHALAMUS REVERSES EARLY LIFE STRESS-INDUCED DEFICITS IN MOTIVATIONAL DRIVE

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ABSTRACT

Altered motivated behavior is observed in a number of psychiatric conditions. One well-characterized antecedent to the development of mood disorders in adulthood is exposure to early life stress (ELS). A central brain site controlling motivated behavior is the lateral hypothalamus (LH). Here we examined the effect of ELS on the motivation to self-administer sucrose using progressive ratio responding (PR). We tested whether chemogenetic activation of LH circuits could modify sucrose responding in ELS rats and examined the impact on LH cell populations using Fos-mapping. Male rat pups were maternally separated for 0hrs or 3hrs on postnatal days 2-14. During adolescence, rats received bilateral injections of hM3D(Gq) into LH. In adulthood, rats were trained to self-administer sucrose and tested under a PR schedule for their motivation for reward with either 5% DMSO or 5mg/kg clozapine-*N*-oxide. Brains were processed for Fos-protein immunohistochemistry. ELS significantly suppressed lever responding for sucrose under PR conditions indicating a long lasting impact of ELS on motivation circuits. hM3D(Gq) activation of LH recovered this behavior in ELS rats and was associated with a significant increase in Fos-positive orexin and MCH cells. Our study indicates that LH circuits retain the capacity to overcome ELS-induced deficits in motivated behavior.

INTRODUCTION

Early life stress (ELS) is a known antecedent to the development of psychiatric conditions including disorders of mood and emotion in adulthood [1,2]. For example, cardinal symptoms of depression are behavioral despair and low motivational drive towards stimuli that are normally naturally rewarding. To study the brain pathways responsible for the expression of these symptoms in humans, preclinical research in rodents has used maternal separation as a model of ELS because it closely recapitulates the neuroendocrine and behavioral abnormalities observed in humans after early life trauma [3,4]. Such studies have largely focused on the neuroendocrine hypothalamus and associated feedback sites such as the hippocampus [3,5]. Importantly, other hypothalamic areas play a critical role in motivational drive. For example, lesion and electrical stimulation studies have shown that the lateral hypothalamus (LH) is an important substrate for motivated behavior and reward-seeking. Indeed, the LH was identified as one of the first brain areas to support intra-cranial self-stimulation [6,7]. To date however, there have been relatively few studies addressing the impact of ELS on LH circuits and its control of motivated behavior.

The LH contains a number of distinct cell populations including neuropeptide-containing cells such as those that express melanin-concentrating hormone (MCH) or orexin (hypocretin). The LH also contains populations of GABAergic and glutamatergic interneurons, some of which act locally within the LH. For example, Jennings and colleagues showed that optogenetic stimulation of a specific population of GABAergic neurons in the LH profoundly increased food-seeking behaviors [8,9]. These GABAergic neurons represent a distinct population in the LH that do not co-express orexin or MCH and likely project directly to the ventral tegmental area (VTA). However, while several previous studies have identified that LH

manipulations can enhance reward-seeking beyond normal (baseline) levels, it is unclear whether manipulations targeting LH can overcome a state of low motivational drive produced by exposure to ELS.

Towards this end, we have previously reported that after exposure to a psychological stressor (restraint) in adulthood, ELS-exposed rats display lower levels of activity in the open field [10]. Notably, no changes in anxiety-like behavior were observed in ELS versus control animals. With respect to LH circuit function, orexin neurons appeared hyporesponsive to restraint stress, but the impact on other LH cell populations, including MCH or GABA-interneurons, was not assessed. Based on this previous data, we hypothesized that ELS would reduce motivational drive to a sucrose reward under progressive ratio (PR) conditions. Our data confirmed that ELS provoked a state of altered motivational drive with animals displaying lower motivation to lever press for sucrose on a PR schedule of reinforcement compared to controls. We next sought to test whether chemogenetic or designer receptors exclusively activated by designer drugs (DREADD) activation of LH circuits could positively modify sucrose responding in ELS animals. We also characterized the impact of DREADD on LH neurons including orexin and MCH-positive cells given their importance to reward-seeking.

RESULTS

Experiment one: Effect of chronic ELS on sucrose self-administration and PR responding in adulthood (n=29)

Sucrose self-administration. For experimental timeline, please refer to Fig 1. Analysis of active lever presses during fixed ratio 1 (FR1) training revealed no significant effect of Neonatal Treatment (No ELS, ELS) ($F_{(1,27)}$=0.3, p=0.857; Fig 2A). Fixed ratio 3 (FR3) active lever responding approached significance ($F_{(1,27)}$=3.1, p=0.090; Fig 2B) with ELS rats exhibiting a reduced number of active lever presses throughout FR3 training. There was no significant effect of Neonatal Treatment on inactive lever responding in FR3 training ($F_{(1,27)}$=0.5, p=0.491; Fig S1A). Interestingly, analysis of active lever presses under a PR schedule of reinforcement revealed a significant interaction between PR Day (1-5) and Neonatal Treatment ($F_{(4,76)}$=2.7, p=0.035). There was also a significant effect of Neonatal Treatment on the number of active lever presses during PR responding ($F_{(1,19)}$=10.5, p=0.004) with ELS rats having a significantly lower number of active lever presses (Fig 2C). Neonatal Treatment had no significant effect on the number of inactive lever responses ($F_{(1,19)}$=2.8, p=0.108; Fig S1B). An analysis of PR breakpoint revealed a significant interaction between Neonatal Treatment and PR Day ($F_{(4,76)}$=3.6, p=0.009). There was also an effect of Neonatal Treatment on PR breakpoint ($F_{(1,19)}$=11.7, p=0.003) with ELS rats having a significantly lower breakpoint than No ELS rats (Fig 2D).

Forced swim test. There was no significant effect of Neonatal Treatment on the time spent climbing, swimming or time in immobility in the forced swim test ($F_{(1,12)}$=0.5, p=0.483; $F_{(1,12)}$=3.6, p=0.08; $F_{(1,12)}$=0.8, p=0.391; Fig 2E, F, G).

Experiment two: Effect of chemogenetic activation of the LH on chronic ELS-induced deficits in motivational drive (n=23).

Sucrose self-administration. For experimental timeline, please refer to Fig 3. Based on the anhedonic-like behavior observed in ELS-exposed rats in experiment one, experiment two aimed to determine whether chemogenetic activation of the LH prior to a final PR test session could reverse this deficit. An analysis of active lever presses during PR revealed an interaction between Drug (Vehicle, CNO) and Neonatal Treatment that approached significance ($F_{(1,20)}$=4.5, p=0.074). There was an effect of Neonatal Treatment on the number of active lever presses during PR responding ($F_{(1,20)}$=6.0, p=0.049). Subsequent one-way ANOVAs revealed no difference between ELS or No ELS rats when treated with CNO ($F_{(1,21)}$=0.1, p=0.746) however when treated with Vehicle, ELS rats exhibited significantly lower lever presses compared to No ELS ($F_{(1,21)}$=4.3, p=0.050; Fig 4A). There was a significant effect of Drug on PR active lever presses ($F_{(1,20)}$=16.3, p=0.001) with CNO increasing lever responding for sucrose (Fig 4A) without affecting inactive lever responding ($F_{(1,20)}$=1.9, p=0.185; Fig S1C). An analysis of PR breakpoint revealed a significant effect of Drug on PR breakpoint ($F_{(1,20)}$=14.5, p=0.001) with CNO resulting in an increased breakpoint for sucrose (Fig 4B). A main effect of Neonatal Treatment approached significance ($F_{(1,20)}$=5.1, p=0.063) with subsequent ANOVAs revealing a lower breakpoint in the ELS-Vehicle group compared to No ELS-Vehicle ($F_{(1,21)}$=3.6, p=0.073; Fig 4B).

Body weight, food and water consumption. There was no effect of Neonatal Treatment on postnatal day (PND) 86 body weight ($F_{(1,21)}$=0.6, p=0.817; Fig 4C). There was also no significant difference between ELS and No ELS rats in food or

water consumption throughout the sucrose self-administration period ($F_{(1,21)}$=0.7, p=0.398; $F_{(1,21)}$=1.5, p=0.228; Fig 4D, E).

Fos-mCitrine immunohistochemistry. Figure 5A illustrates representative hM3D(Gq) viral expression observed in the LH. Supplementary figures S2 and S3 illustrate the extent of hM3D(Gq) viral expression in the LH. There was no effect of Neonatal Treatment and Drug on the number of mCitrine-positive cells in the LH ($F_{(1,20)}$=3.5, p=0.074) or dorsomedial hypothalamus (DMH) ($F_{(1,20)}$=4.2, p=0.053) (Table 1). Although there appeared to be a significant interaction between the two independent variables on the number of mCitrine-positive cells in the DMH, the total number of these cells was small (<20) unlikely having an effect on behavior. In the LH, there was a near significant effect of Neonatal Treatment with ELS rats having overall increases in the percentage of Fos-positive mCitrine cells ($F_{(1,20)}$=4.3, p=0.052). Additionally, CNO significantly increased the percentage of Fos-positive mCitrine cells ($F_{(1,20)}$=91.9, p<0.0001; Fig 5B). In the DMH, there was no effect of Neonatal Treatment on the percentage of Fos-positive mCitrine cells ($F_{(1,20)}$=0.4, p=0.555). However, of the small number of mCitrine cells present in the DMH, CNO-treated rats had an increased number that were Fos-positive ($F_{(1,20)}$=10.2, p=0.012; Fig 5B).

Fos-orexin immunohistochemistry. There was no effect of Neonatal Treatment and Drug on the number of orexin cells in the LH ($F_{(1,20)}$=1.2, p=0.294) or DMH ($F_{(1,20)}$=0.2, p=0.629) (Table 1). In the LH, there was no significant effect of Neonatal Treatment ($F_{(1,20)}$=0.0, p=0.993) on the percentage of Fos-positive orexin neurons (Fig 5C). However, there was a significant effect of Drug ($F_{(1,20)}$=83.7, p<0.0001) with CNO-treated rats having a greater percentage of Fos-positive orexin cells compared to Vehicle-treated rats (Fig 5C). In the DMH, there was no significant interaction

between Drug and Neonatal Treatment on the percentage of Fos-positive orexin cells ($F_{(1,20)}$=0.1, p=0.747) (Fig 5C).

Fos-MCH immunohistochemistry. There was also no effect of Neonatal Treatment and Drug on the number of MCH neurons in the LH ($F_{(1,20)}$=0.1, p=0.770) DMH ($F_{(1,20)}$=0.8, p=0.392) (Table 1). In the LH, there was no significant difference across Neonatal Treatment groups on the percentage of Fos-positive MCH cells ($F_{(1,20)}$=3.8, p=0.067; Fig 5D). Interestingly, CNO-treated rats had overall increases in the percentage of Fos-positive MCH cells compared to Vehicle-treated rats ($F_{(1,20)}$=85.9, p<0.0001; Fig 5D). In the DMH, there was no significant interaction between Neonatal Treatment and Drug on the percentage of Fos-positive MCH neurons ($F_{(1,20)}$=0.0, p=0.910; Fig 5D).

Fos-protein immunohistochemistry. An analysis of the number of Fos-positive cells that were orexin and MCH negative in the LH revealed an interaction between Drug and Neonatal Treatment ($F_{(1,20)}$=6.1, p=0.023). There was a significant main effect of Neonatal Treatment ($F_{(1,20)}$=15.8, p=0.01) with the ELS-CNO group having an increased number of Fos-positive cells compared to the No ELS-CNO group (p=0.006; Fig 5E). There was also a significant effect of Drug on the number of Fos-positive cells in the LH ($F_{(1,20)}$=485.2, p<0.0001) with CNO resulting in increased Fos counts compared to Vehicle controls (Fig 5E). In the DMH, there was no significant interaction between Neonatal Treatment and Drug on the number of Fos-positive cells in the DMH ($F_{(1,20)}$=1.1, p=0.310; Fig 5E).

HA-MCH-orexin immunohistochemistry. Fig 6 illustrates the absence of colocalization between hM3D(Gq) virally transduced cells and orexin (n=102±4 orexin cells, n=3±1 HA+orexin cells) or MCH (n=134±22 MCH cells, n=7.5±1 HA+MCH cells) in the LH.

DISCUSSION

The present study provides evidence that exposure to ELS promotes deficits in motivated responding for sucrose that are long lasting and can be observed in adulthood. These findings support a hypothesis that the brain circuits controlling motivated behavior are vulnerable to long lasting remodelling by ELS. Importantly, using hM3D(Gq) DREADD targeting the LH, we found that the designer ligand CNO recovered responding for sucrose in ELS rats to levels equivalent to No ELS controls. CNO-treatment was associated with an increased number of Fos-positive cells in the LH including MCH and orexin and putative GABA and glutamatergic interneurons. These data indicate that selective pharmacological targeting of LH cells can restore function in circuits impaired by ELS exposure.

We have previously shown that ELS exposure produced hypoactivity in the open field and reduced stress-reactivity including activation of neuroendocrine cells and LH orexin neurons after adult restraint stress [10]. Here we aimed to determine the impact of ELS on motivated responding for sucrose in adulthood. We observed that ELS rats displayed reduced PR lever pressing for sucrose compared to No ELS controls. In contrast, ELS did not alter forced swim behavior. Thus, in our hands, maternal separation produces a phenotype in male rats that more closely resembles deficits in motivational drive, rather than frank behavioral despair detected in the forced swim test.

Notably, no differences in FR1 or FR3 responding for sucrose were observed between ELS and No ELS groups suggesting that baseline taste preference or learning differences did not influence PR responding. Indeed, even on day one of PR testing, ELS and No ELS rats were equally motivated to lever press for sucrose with differences in motivated responding only evident on succeeding days. These findings

are remarkably consistent with results reported by Rüedi-Bettschen and colleagues [11] who showed that ELS results in reduced PR responding for a 7% sucrose reward, an effect that was only seen after repeated PR testing. Furthermore, these authors also reported no significant differences in forced swim behavior between ELS and No ELS rats.

Our studies demonstrate that transduction of LH cells with the hM3D(Gq) DREADD increased lever responding for sucrose under PR conditions in both ELS and No ELS groups. With respect to the impact of ELS on motivated responding for sucrose, hM3D(Gq) DREADD activation in LH recovered responding to levels seen in the No ELS group. These data are noteworthy at several levels. First, they show that motivation to lever press for sucrose can be enhanced by LH activation and second, capacity remains within the LH circuitry to overcome a state of low motivational drive produced by ELS. Together these data indicate that ELS produces a long lasting remodelling of LH circuitry but, importantly, pharmacological manipulations targeting LH cell populations can recover these behavioral deficits, even in adulthood.

Counts of Fos-positive cells and Fos-positive LH cells that were orexin and MCH negative revealed that ELS increased activity in the LH, but only after CNO administration. This pattern of Fos activity corresponded to the reduction in motivated responding for sucrose, reflecting an altered sensitivity of LH following ELS. We have previously shown that orexin cell reactivity was reduced compared to No ELS controls in response to psychological stress in adulthood [10]. Somewhat surprisingly therefore, we found here that the activity of orexin cells was similar between ELS and control rats after sucrose PR – even though PR responding was lower in ELS rats. Notably, very few Fos-positive MCH cells were observed in the LH of ELS and No

ELS rats suggesting that this population of cells is not strongly impacted by maternal separation. It is relevant to note that there were differences in study design between this and our previous report [10] that may account for these discrepancies. Most significantly, hypoactivity of orexin cells after ELS in our previous work was only unmasked after exposure to restraint stress in adulthood. Further, here rats were trained for sucrose self-administration and although low under PR conditions, sucrose consumption may have masked activity differences in LH circuitry caused by ELS. Fos-mapping has well known limitations, including temporal and resolution, making future studies using electrophysiological recordings a logical next step to detect ELS remodelling of LH circuits.

CNO activation of hM3D(Gq) significantly increased the numbers of Fos-positive cells in the LH including those immuno-positive for MCH and orexin. The recruitment of orexin neurons by DREADD paired with the increased lever responding for sucrose is consistent with the accumulating evidence of a role for this system in motivated responding [12-16]. For example, Choi et al. [17] found that 5nmole intracerebroventricular (icv) orexin-A administration increased PR responding for sucrose pellets while systemic injections of 10mg/kg SB-334867, an OX1R antagonist, reduced PR responding. Although CNO also increased the recruitment of MCH neurons, the overall numbers of Fos-positive MCH neurons were low, making it unlikely that their contribution to increased motivation was behaviorally relevant. This finding is consistent with work by Petrovich and colleagues [18] who demonstrated minimal MCH and robust orexin neuron recruitment to a learned food cue.

In our study, orexin and MCH neurons only represented 20-30% of the total population of cells demonstrating increased Fos-protein after CNO (Fig 5E). This suggests that a significant percentage of Fos-positive cells in LH after DREADD

activation by CNO are glutamatergic or GABAergic LH cell populations. Activation of *Vgat* neurons in LH can drive both consummatory and reward behaviors [19]. In an important study using optogenetics, Barbano et al. [20] revealed that the frequency of stimulation of LH *Vgat*→VTA neurons codes for consummatory versus reward behaviors. Taken together with studies showing that orexin neurons mediate reward-seeking behavior, it is possible that parallel, independent circuits from LH→VTA can promote reward-seeking behaviors ie. direct LH GABA→VTA and LH orexin→VTA pathways [20,21]. Importantly, a role for local glutamatergic neurons in enhancing or synchronising LH output cannot be ruled out [9,22-25].

In the present study we used a pan-neuronal AAV5-hSyn-hM3D(Gq) DREADD rather than the AAV-CAMKII which, based on studies in other brain areas, would have biased DREADD transduction to glutamatergic neurons [26,27]. CNO resulted in increased activity, as assessed by Fos-protein, in DREADD-positive cells (Fig 5B). However, immunohistochemical detection for HA, orexin and MCH revealed very little colocalization with these major LH cell types (Fig 6). While this may be due to the sensitivity of 'endogenous' or immunohistochemical detection methods, these findings suggest that the DREADD may have preferentially transduced non-orexin and non-MCH-expressing cell populations. Unfortunately, our attempts to immunolabel for GAD67 in LH were unconvincing, despite the known population of GAD67-expressing neurons in the area [9,22-25]. Thus, it is likely that our viral injections transduced GABA and glutamatergic interneuron populations in the LH [9,19].

In conclusion, we show here that chemogenetic activation of LH circuits can enhance motivated responding for sucrose under PR conditions and overcome a state of low motivational drive produced by ELS. Interestingly, AAV5-hSyn DREADD

appeared to promote this behavior by transduction of intra-hypothalamic neurons that directly project to the LH or act via local circuits to influence the action of orexin and to a lesser extent MCH neurons. Overall these data have important implications clinically because they show that while LH circuitry can be maladapted by ELS, selective pharmacological activation of appropriate cell types in LH can overcome low motivated arousal; a debilitating symptom of neuropsychiatric diseases such as depression. While further work is required to dissect the specific LH circuits that mediate these effects, such advances may aid in developing more effective treatments for depressive illnesses.

METHODS AND MATERIALS

Ethics statement

All experiments performed were approved by the University of Newcastle Animal Care and Ethics Committee, and carried out in accordance with the National Health and Medical Research Council Australian Code of Practice for the care and use of animals for scientific practice.

Animals

12 experimentally naïve Wistar dams were obtained from the Animal Resources Centre, Canning Vale, Western Australia, and bred with six experimentally naïve males at the University of Newcastle. A total of 52 male rats were included in the study. On PND1, the day of birth, animals from each litter were randomly allocated to the ELS group (3hr maternal separation) or the No ELS group. On PND21, rats were weaned to two rats per cage (Able Scientific, Sydney). Food (Rat and Mouse Pellets, Glen Forest, Western Australia) and water were available ad libitum and rats were maintained on reverse 12hr light/dark cycle (lights off at 0700hrs).

Early life stress (ELS)

The maternal separation procedure was performed as described by James et al. [10]. Briefly, from PND2-14, litters in the ELS condition were removed from their home cage and individually separated in an alternate temperature controlled room (30-34ºC) for 3hrs/day, from 9am–12pm. Pups in the No ELS condition remained undisturbed during this period except for weekly weighing.

Surgery

All surgeries were performed on rats between PND42-44 (~200grams). Rats were anesthetized with isofluorane prior to placing them in the stereotaxic frame (Stoelting instruments). Rats were injected intramuscularly with 0.3mL of a broad-spectrum

antibiotic (150mg/mL procaine penicillin, 112.5mg/mL benzathine penicillin; Norbrook Laboratories, UK) and subcutaneously with 0.2mL of a 50mg/mL solution of carprofen (Norbrook Laboratories, UK).

Viral injections. rAAV5/hSyn-HA-hM3D(Gq)-IRES-mCitrine (UNC vector core, Lot# AV4619F) was bilaterally injected into the LH (AP: -2.4, ML: ±1.9, DV: -8.4). 1μl was injected over 4mins and the needle was left in place for 2mins. Injections were made using a 2μl Hamilton Neuros syringe (30G) attached to a Stoelting Quintessential Stereotaxic Injector pump (Cat# 53311).

Behavioral procedures

Sucrose self-administration. On PND70-75, all animals were trained to self-administer sucrose (10% w/v) in daily 30min sound-attenuated operant chamber sessions under a FR1 administration schedule (Med Associates, VT, USA). During FR1 training, responding on the active (right) lever resulted in the delivery of 0.1ml of sucrose into a receptacle followed by a 4sec light stimulus located above the active lever, indicating the availability of sucrose. The inactive (left) lever was not extended during FR1 training. FR1 sessions continued for eight days where on the first four days of FR1 training, all rats had restricted access to water, 2hrs/day (from 3-5pm), to facilitate acquisition of operant conditioning. Following FR1 training, rats progressed to a FR3 schedule for four days where both the active and inactive (left) levers were presented. Responding on the inactive lever was recorded but had no scheduled consequence. During the final stage of sucrose self-administration, PR responding was assessed during five 90min sessions using a reinforcement schedule where the number of lever presses required for sucrose reward delivery increased by 1 following each reward, ie. 1, 2, 3, 4, 5, etc. [28,29]. Breakpoint was defined as the highest response

ratio completed (ie. total number of rewards received) throughout the PR session. All behavioral testing took place between 9am and 3pm daily.

Forced swim test. A subgroup of animals was exposed to a forced swim test immediately prior to the last PR session. Briefly, rats were placed in 40cm of tepid water (25-30°C) and swimming, immobility and climbing behavior were recorded for 10mins.

Sacrifice and tissue harvesting.

Two hours following the initiation of the final PR test, rodents were anaesthetized with sodium pentobarbitone (1ml intraperitoneal, i.p, Virbac, Australia) and perfused with 200mL of 0.1M Phosphate Buffered Saline (PBS) followed by 500mL of 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde at 4ºC for 2hrs, then transferred to 30% sucrose in 0.1M PBS for 48hrs at 4ºC. Brains were frozen on dry ice and stored at -80ºC until sectioning.

Dual Fos-protein and mCitrine immunohistochemistry. Serial 40μm thick coronal sections of the forebrain were cut using a cryostat (Leica Biosystems CM1900, North Ryde, NSW, Australia) and a 1-in-4 series of hypothalamic sections (Bregma -2.60 to -2.92) were processed for immunohistochemical detection of Fos-protein and mCitrine. Tissue was incubated for 48hrs in a 1:8000 primary Fos antibody concentration (rabbit monoclonal, 5348S, Cell Signaling Technology, MA, USA) followed by a two hour incubation in a 1:1000 concentration donkey anti-rabbit secondary (711-065-152, Jackson IR, PA, USA). After visualisation of Fos-protein with glucose oxidase, sections were incubated for 72hrs in a 1:2000 primary anti-GFP antibody concentration (chicken polyclonal, ab13970, Abcam, MA, USA) followed by a two hour incubation in a 1:400 concentration donkey anti-chicken secondary (703-005-155, Jackson IR, PA, USA).

Dual Fos-protein and orexin immunohistochemistry. A second series of tissue was processed for immunohistochemical detection of Fos-protein and orexin. Briefly, free floating brain sections were incubated for 48hrs at room temperature in a solution of PBS azide containing 0.5% Triton X-100, 2% normal horse serum and a cocktail of primary antibodies (1:2000 c-Fos antibody, rabbit monoclonal, 5348S, Cell Signaling Technology, MA, USA and 1:1000 orexin A antibody, goat polyclonal, sc-8070, Santa Cruz Biotechnology, CA, USA). Sections were then incubated for 2hrs at room temperature in a cocktail of secondary antibodies (1:1000, Alexa Fluor 594 donkey anti rabbit, Invitrogen, and 1:500, AMCA anti-goat, Jackson Immunoresearch). Sections were finally mounted onto gelatin-coated slides and coverslipped with gelvatol.

Dual Fos-protein and MCH immunohistochemistry. An adjacent series of tissue was processed for immunohistochemical detection of Fos-protein and MCH. Free floating sections were incubated for 72hrs at 4°C in a solution of PBS containing 0.5% Triton X-100, 2% normal horse serum and a cocktail of primary antibodies (1:2000 c-Fos antibody, rabbit monoclonal, 5348S, Cell Signaling Technology, MA, USA and 1:1000 MCH antibody, goat polyclonal, sc-14507, Santa Cruz Biotechnology, CA, USA). Sections were then incubated for 3hrs at room temperature in a cocktail of secondary antibodies (1:200, Alexa Fluor 488 donkey anti rabbit, Invitrogen, and 1:200, Alexa Fluor 594 anti-goat, Jackson Immunoresearch).

HA and MCH or orexin immunohistochemistry. A forth series of tissue was processed for hemagglutinin (HA) immunoreactivity, the viral tag fused to the hM3D(Gq) DREADD, with MCH or orexin. All immunohistochemical methods were as described above with HA in place of Fos. A 1:1000 primary HA antibody (mouse

monoclonal, 901513, BioLegend, CA, USA) concentration was used with 1:400 Alexa Fluor 488 anti-mouse (Jackson Immunoresearch) as the secondary.

Neuronal quantification

Tissue was imaged using Olympus cellSens imaging software (version 1.3) under a 10x microscopic objective (Olympus BX51). A 40x microscopic objective was used for HA immunohistochemistry. Images were quantified using iVision (Biovision) software where bilateral counts of Fos, MCH, orexin, HA and GFP were analyzed across Bregma levels (-2.60, -2.76 and -2.92) by one observer, blind to treatment. Dual labeled cells were quantified by merging the two cell images in iVision. Given the spread of the virus, immunohistochemical quantification occurred in two regions of the hypothalamus, the perifornical/lateral hypothalamic area (LH) and the DMH. The LH was defined as the area from the medial side of the fornix to the optic tract and the DMH was defined as the region from the medial side of the fornix to the third ventricle [10, 30, 31]. The total number of Fos-positive cells that were orexin and MCH negative was calculated by subtracting the number of Fos-positive orexin cells and Fos-positive MCH cells from total Fos counts.

Specific experiments

Experiment one: Effect of ELS on depressive-like behavior in adulthood (n=29). Rats were exposed to either ELS or No ELS and remained undisturbed until adulthood. On PND70-75, all rats were trained to self-administer 10% sucrose on FR1, FR3 and PR schedules of reinforcement. Between PND86-91, rats were given one final PR session (PR day five) with a subset of these rats (n=14) being exposed to the forced swim test immediately prior to PR day five. These animals were not included in statistical analysis for PR day five. For experimental timeline, please refer to Fig 1.

Experiment two: Effect of chemogenetic activation of the lateral hypothalamus on ELS-induced deficits in motivational drive (n=23). Rats were exposed to either ELS or No ELS. On PND 42-44, all rats received intracranial hM3D(Gq) viral injections into the lateral hypothalamus. On PND70-75, all rats were trained to self-administer 10% sucrose on FR1, FR3 and PR schedules of reinforcement. Rats received three lots of 5% DMSO in 0.9% saline solution (1mL/kg) habituation injections (i.p) throughout operant training. On PR day five, rats were given either vehicle (5% DMSO; 1mL/kg; i.p) or clozapine-*N*-oxide (CNO dissolved in 5% DMSO; 5mg/kg; i.p; National Institute of Mental Health Chemical Synthesis and Drug Supply Program, RTI International) injections 30mins prior to the PR self-administration session. To ensure the CNO had completely metabolised, rats were given an additional self-administration session the following day, PR day six. On PR day seven, the administration of vehicle or CNO 30mins prior to self-administration was reversed (counterbalanced order) and rats were deeply anaesthetized with sodium pentobarbitone two hours following the initiation of the session and perfused. Brains were then processed for immunohistochemical detection of Fos-protein, orexin, MCH and HA. Food and water consumption was monitored every second day throughout sucrose self-administration training to examine any potential non-specific effects of hM3D(Gq) viral transduction and whether any differences in sucrose self-administration observed were compensated for with normal rat chow or water intake. Both food and water consumption were estimated across all groups by weighing food and water, dividing this number by two to assess consumption per day and dividing this number by the number of animals per cage. For experimental timeline, please refer to Fig 3.

Data Analysis

Statistical analyses were conducted using IBM SPSS V22. Sucrose preference was analysed using an analysis of variance (ANOVA) with the between-subjects factor of Neonatal Treatment (No ELS, ELS) and the within-subjects factor of Session for FR1, FR3 and PR. Forced swim data, body weight and food and water consumption were analysed using between-subjects ANOVAs. The LH DREADD experiment was analysed using the between-subjects factor of Neonatal Treatment and the within-subjects factor of Drug (Vehicle, CNO) with litter size as a covariate. For immunohistochemical analyses, all cell counts were averaged across each animal for each rostrocaudal level of both the LH and DMH. Immunohistochemical data was analysed using a two-way between-subjects ANOVA comparing Neonatal Treatment and Drug. The reactivity of orexin, MCH and GFP neurons was assessed using the percentage of orexin, MCH and GFP cells expressing Fos-protein. All figures are presented as mean + SEM.

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FUNDING AND DISCLOSURE

All authors report no conflicts of interest.

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AUTHOR CONTRIBUTIONS

EJC and CVD designed research; EJC, CSM and CDA performed research; EJC, CVD, CSM and JWY analyzed data; all authors wrote and reviewed the manuscript.

FIGURE LEGENDS AND TABLE

Figure 1. Schematic illustration of the experimental design for experiment 1. Postnatal day (PND); fixed ratio 1 or 3 (FR1, FR3); progressive ratio (PR).

Figure 2. Effect of early life stress (ELS) on sucrose operant responding and forced swim behavior. (**A, B**) There was no effect of ELS on the number of lever presses for sucrose under fixed ratio (FR) 1 and 3 schedules of reinforcement. No ELS: n=14; ELS: n=15. (**C, D**) ELS resulted in a significant reduction in the number of active lever presses and the breakpoint for sucrose under a PR schedule of reinforcement. Bar graphs represent average lever responding and breakpoint over the 5 day PR task. No ELS: n=14; ELS: n=15. (**E, F, G**) ELS resulted in no significant differences in forced swim behavior. No ELS: n=7; ELS: n=7. *p<0.05; **p<0.01.

Figure 3. Schematic illustration of the experimental design for experiment 2. Following early life maternal separation, rats received bilateral injections of hM3D(Gq) virus into the LH during adolescence (postnatal days (PND) 42-44). Rats were then allowed to self-administer sucrose from PND70-85 followed by progressive ratio testing after vehicle or clozapine-*N*-oxide (CNO) injections. Fixed ratio 1 or 3 (FR1, FR3); progressive ratio (PR); intraperitoneal, i.p.

Figure 4. Effect of early life stress (ELS) and lateral hypothalamic (LH) chemogenetic activation on sucrose operant responding, body weight, food and water consumption. (**A**) ELS resulted in reduced lever presses for sucrose under vehicle conditions. clozapine-*N*-oxide (CNO) increased lever responding for sucrose

in ELS rats equivalent to No ELS levels. **(B)** A similar trend was seen for breakpoint. No ELS - Vehicle: n=10; ELS - Vehicle: n=13; No ELS - CNO: n=10; ELS - CNO: n=13. *p<0.05 No ELS – Vehicle versus ELS - Vehicle. ***p=0.001 Vehicle-treated rats versus CNO-treated rats. (**C, D, E**) ELS and LH viral transduction had no effect on final body weight (postnatal day (PND) 86) and food or water consumption over the sucrose self-administration period.

Figure 5. Effect of early life stress (ELS) and chemogenetic lateral hypothalamic (LH) activation on Fos-protein expression, orexin cell reactivity and melanin-concentrating hormone (MCH) neuron reactivity. (**A**) Representative image of hM3D(Gq) viral expression (HA-tag immunostain) in the LH. Scale bars, 200μm and 20μm respectively. Third ventricle (3V); optic tract (OT); mammillary tract (MT); fornix (F). (**B**) Photomicrograph of Fos (black) and GFP (brown) cells. clozapine-*N*-oxide (CNO) resulted in a significantly increased percentage of Fos-positive GFP cells in the LH and DMH. No ELS - Vehicle: n=2; ELS - Vehicle: n=2; No ELS - CNO: n=2; ELS - CNO: n=2. **p<0.01, ***p<0.0001 Vehicle-treated rats versus CNO-treated rats**.** Scale bar, 20μm. **(C)** Photomicrograph of Fos-positive orexin cells. CNO resulted in significantly increased numbers of Fos-positive orexin neurons in the LH but not the DMH. No ELS - Vehicle: n=5; ELS - Vehicle: n=7; No ELS - CNO: n=5; ELS - CNO: n=6. ***p<0.001 Vehicle-treated rats versus CNO-treated rats. Scale bar, 50μm. **(D)** Photomicrograph of dual Fos-MCH immunohistochemistry. CNO resulted in significantly greater numbers of Fos-positive MCH neurons in both the LH but not the DMH. No ELS - Vehicle: n=5; ELS - Vehicle: n=7; No ELS - CNO: n=5; ELS - CNO: n=6. ***p<0.001 Vehicle-treated rats versus CNO-treated rats. Scale bar, 50μm. **(E)** ELS resulted in a significant increase in the number of Fos-

positive neurons in the LH (that were orexin and MCH negative) under CNO-treatment conditions. Additionally, CNO significantly increased the number of Fos-positive cells in the LH. There were no effects of Neonatal Treatment or Drug on the number of Fos-positive cells in the dorsomedial hypothalamus (DMH). No ELS - Vehicle: n=5; ELS - Vehicle: n=7; No ELS - CNO: n=5; ELS - CNO: n=6. **p<0.01 No ELS - CNO versus ELS - CNO. ***p<0.001 Vehicle-treated rats versus CNO-treated rats. Scale bar, 200μm.

Figure 6. Evidence of limited hM3D(Gq) colocalization with neuropeptide containing cell populations in the lateral hypothalamus (LH). (A) Representative photomicrograph of the LH demonstrating the lack of colocalization between HA (green; tag for hM3D(Gq) DREADD) and MCH (red) immunolabeling (n=134±22 MCH cells, n=7±1 HA+MCH cells). **(B)** Representative photomicrograph of the LH demonstrating the lack of colocalization between HA (green) and orexin (blue) immunolabeling (n=102±4 orexin cells, n=3±1 HA+orexin cells). n=2 CNO-treated rats. Scale bar, 20μm.

Treatment	Region	Orexin number	MCH number	hM3D(Gq) number
No ELS - Vehicle (n = 5)	LH	127.6 ± 4.9	197.2 ± 7.9	46.3 ± 6.1
	DMH	28.2 ± 1.9	45.9 ± 6.7	9.8 ± 1.6
ELS – Vehicle (n = 7)	LH	139.9 ± 6.0	182.9 ± 14.2	66.6 ± 8.0
	DMH	29.3 ± 1.9	24.0 ± 4.4	13.3 ± 2.5
No ELS – CNO (n = 5)	LH	125.2 ± 4.9	202.0 ± 6.7	82.9 ± 12.4
	DMH	29.4 ± 2.5	42.4 ± 8.1	16.7 ± 1.6
ELS – CNO (n = 6)	LH	148.3 ± 4.9	193.4 ± 7.6	70.2 ± 7.3
	DMH	32.6 ± 1.9	31.9 ± 8.2	10.3 ± 3.4

Table 1. Orexin, MCH and hM3D(Gq) cell expression in each subregion of the hypothalamus. Data presented as average number of orexin, MCH or GFP cells (as a marker of mCitrine) ± standard error mean. Early life stress (ELS); clozapine-*N*-oxide (CNO); lateral hypothalamus (LH); dorsomedial hypothalamus (DMH).

Figure 1

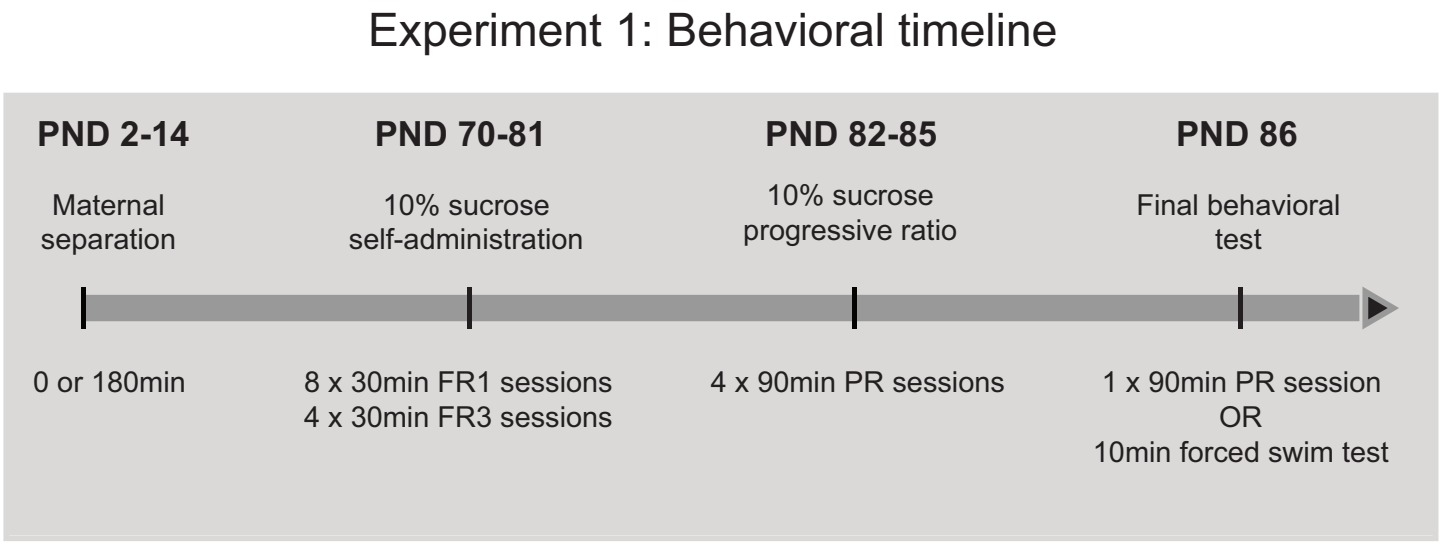

Experiment 1: Behavioral timeline
PND 2-14
Maternal separation
0 or 180min
PND 70-81
10% sucrose self-administration
8 x 30min FR1 sessions
4 x 30min FR3 sessions
PND 82-85
10% sucrose progressive ratio
4 x 90min PR sessions
PND 86
Final behavioral test
1 x 90min PR session
OR
10min forced swim test

Figure 2

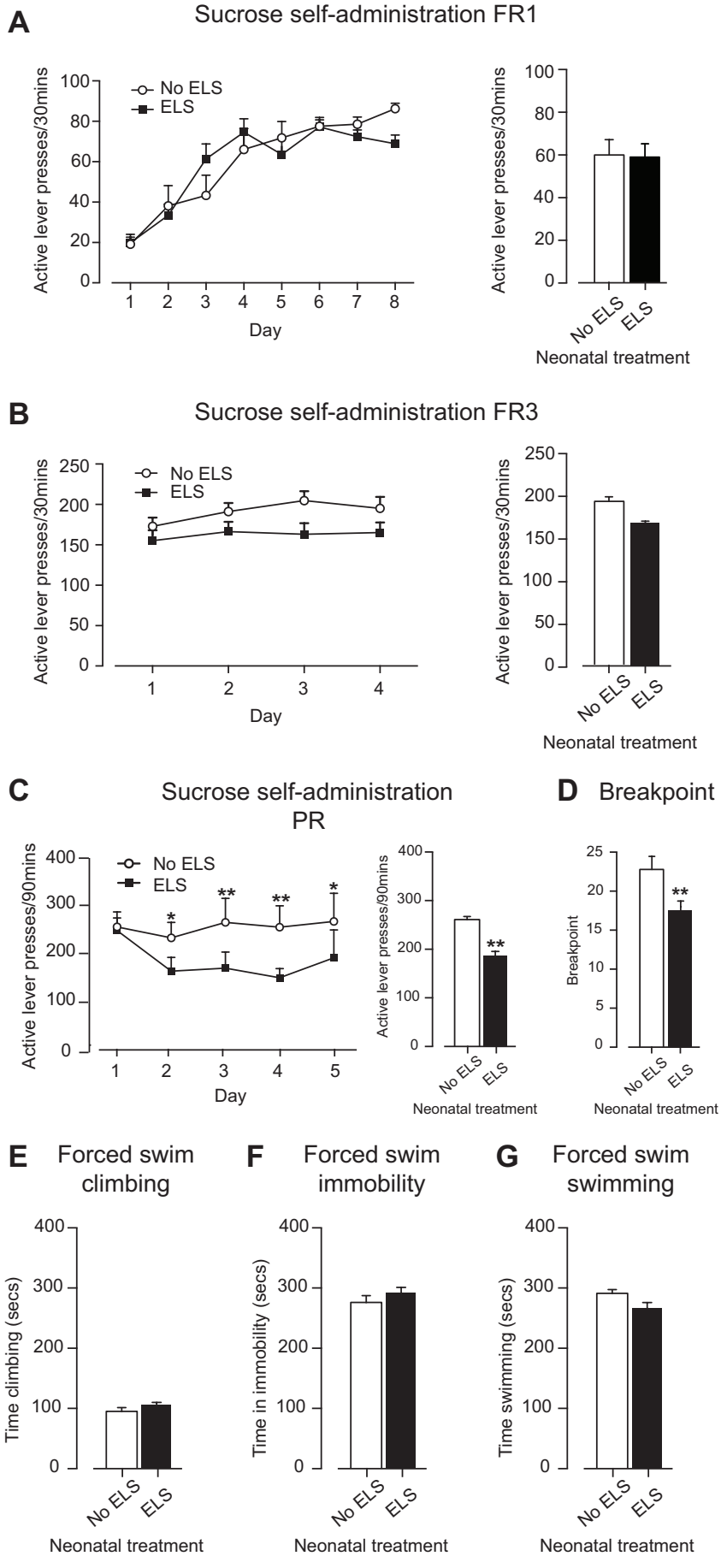

A
Sucrose self-administration FR1
No ELS
ELS
Active lever presses/30mins
Day
Neonatal treatment
B
Sucrose self-administration FR3
C
Sucrose self-administration PR
Active lever presses/90mins
D
Breakpoint
E
Forced swim climbing
Time climbing (secs)
F
Forced swim immobility
Time in immobility (secs)
G
Forced swim swimming
Time swimming (secs)

Figure 3

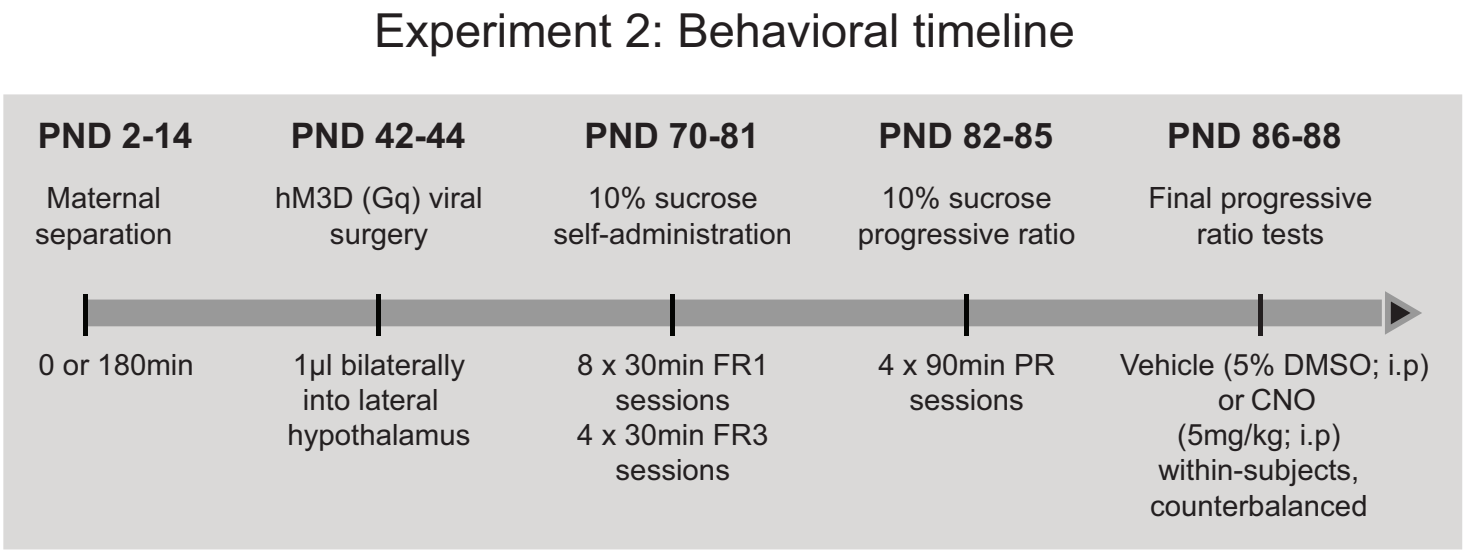

Experiment 2: Behavioral timeline
PND 2-14
Maternal separation
0 or 180min
PND 42-44
hM3D (Gq) viral surgery
1µl bilaterally into lateral hypothalamus
PND 70-81
10% sucrose self-administration
8 x 30min FR1 sessions
4 x 30min FR3 sessions
PND 82-85
10% sucrose progressive ratio
4 x 90min PR sessions
PND 86-88
Final progressive ratio tests
Vehicle (5% DMSO; i.p) or CNO (5mg/kg; i.p) within-subjects, counterbalanced

Figure 4

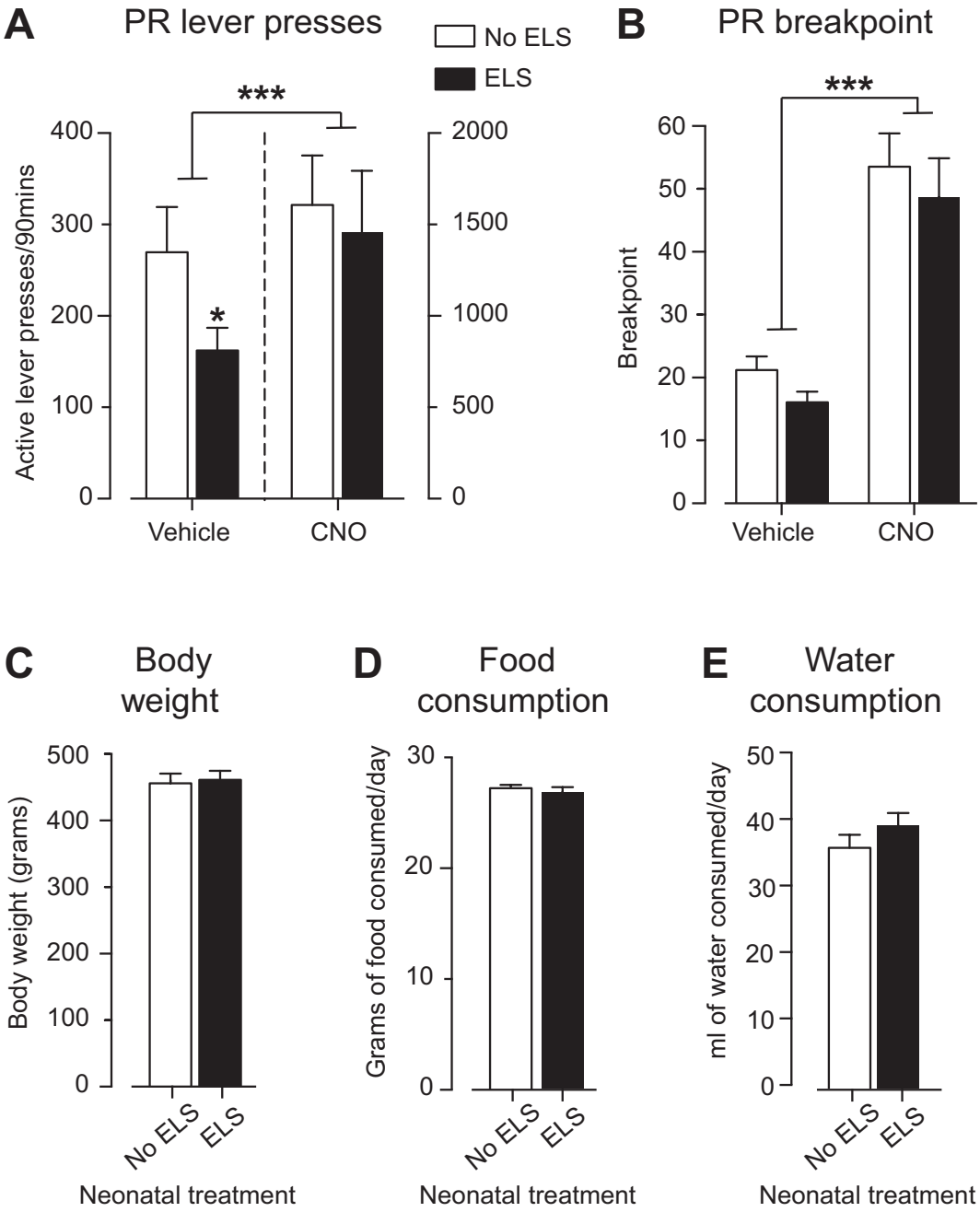

A
PR lever presses
No ELS
ELS
***
*
Active lever presses/90mins
400
300
200
100
0
2000
1500
1000
500
0
Vehicle
CNO
B
PR breakpoint
***
Breakpoint
60
50
40
30
20
10
0
Vehicle
CNO
C
Body weight
Body weight (grams)
500
400
300
200
100
0
No ELS
ELS
Neonatal treatment
D
Food consumption
Grams of food consumed/day
30
20
10
0
No ELS
ELS
Neonatal treatment
E
Water consumption
ml of water consumed/day
50
40
30
20
10
0
No ELS
ELS
Neonatal treatment

Figure 5

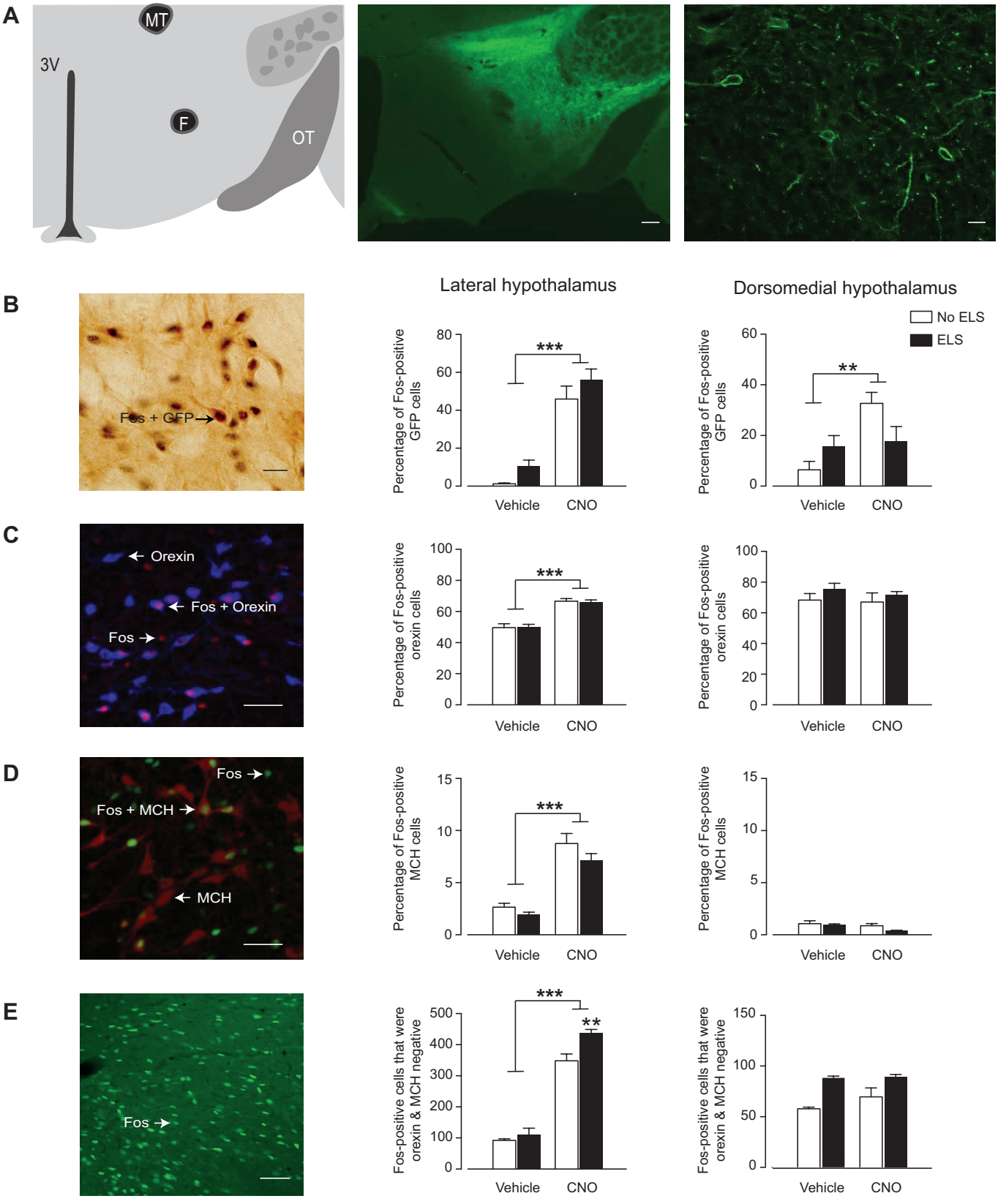
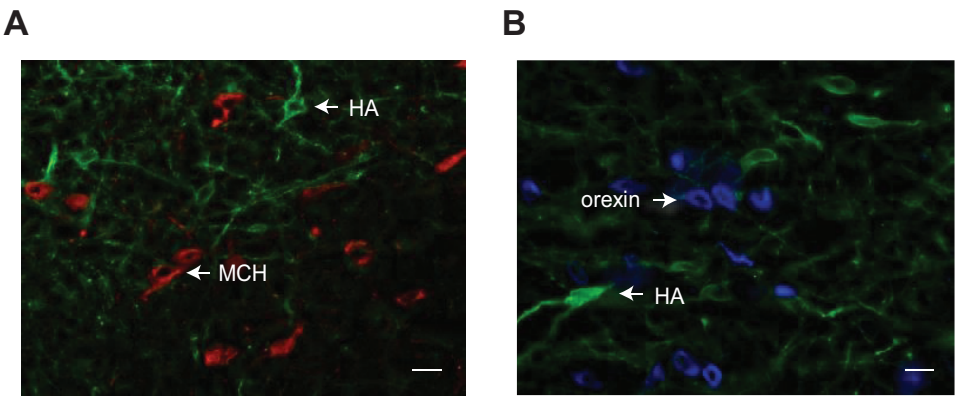

A
MT
3V
F
OT
B
Fos + GFP
Lateral hypothalamus
Dorsomedial hypothalamus
No ELS
ELS
Percentage of Fos-positive GFP cells
***
**
Vehicle
CNO
C
Orexin
Fos + Orexin
Fos
Percentage of Fos-positive orexin cells
D
Fos
Fos + MCH
MCH
Percentage of Fos-positive MCH cells
E
Fos
Fos-positive cells that were orexin & MCH negative

Figure 6



SUPPLEMENTARY FIGURES

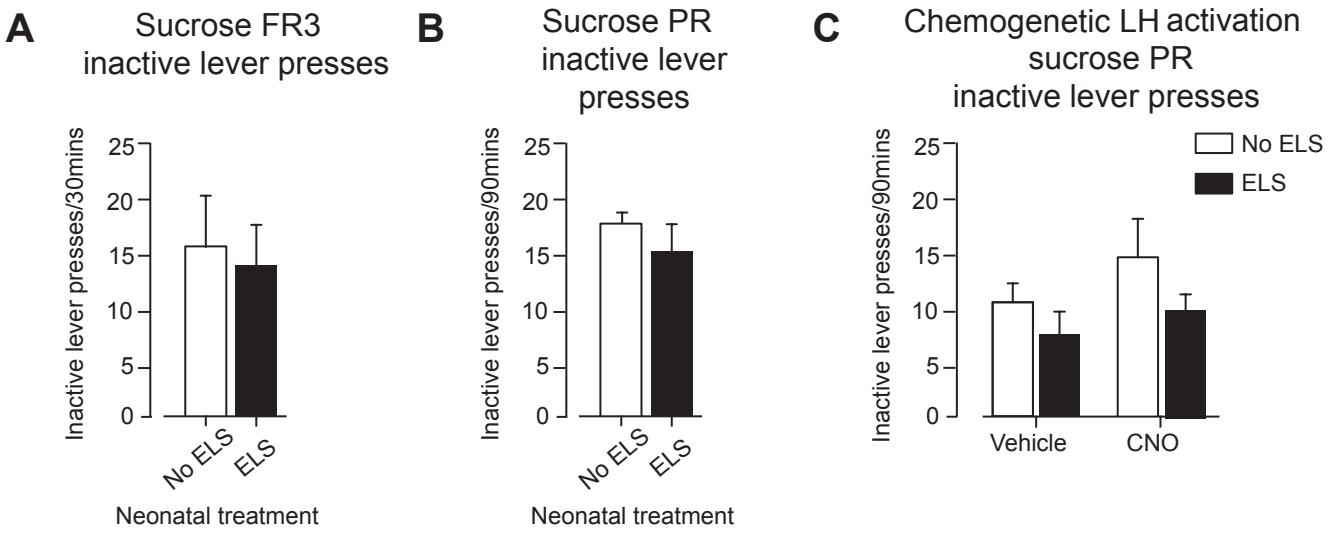


Figure S1. Effect of early life stress (ELS) and chemogenetic lateral hypothalamus activation on inactive lever presses for sucrose (**A, B**) There was no effect of ELS on the number of inactive lever presses under a fixed ratio 3 (FR3) or progressive ratio (PR) schedule of reinforcement for sucrose. No ELS: n=14; ELS: n=15. (**C**) There was no effect of CNO on the number of inactive lever responses for sucrose. No ELS - Vehicle: n=10; ELS - Vehicle: n=13; No ELS - CNO: n=10; ELS - CNO: n=13. Lateral hypothalamus (LH).

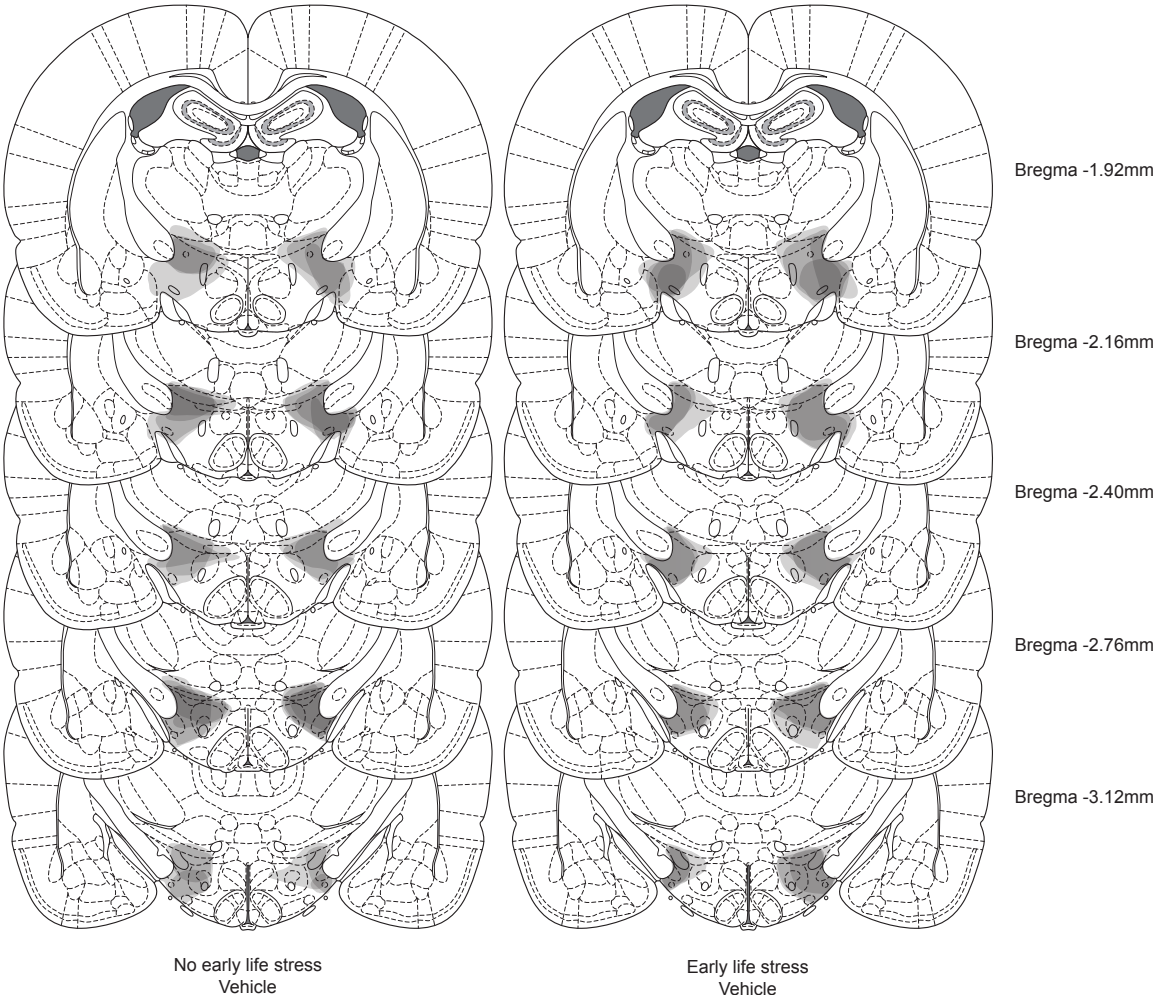


Figure S2. Vehicle-treated rat viral expression. Extent of hM3D(Gq) viral expression in the lateral hypothalamus across the two vehicle treatment groups. Minimum expression is represented by light grey and maximum expression is represented by dark grey. Coronal slices adapted from Paxinos & Watson[31].

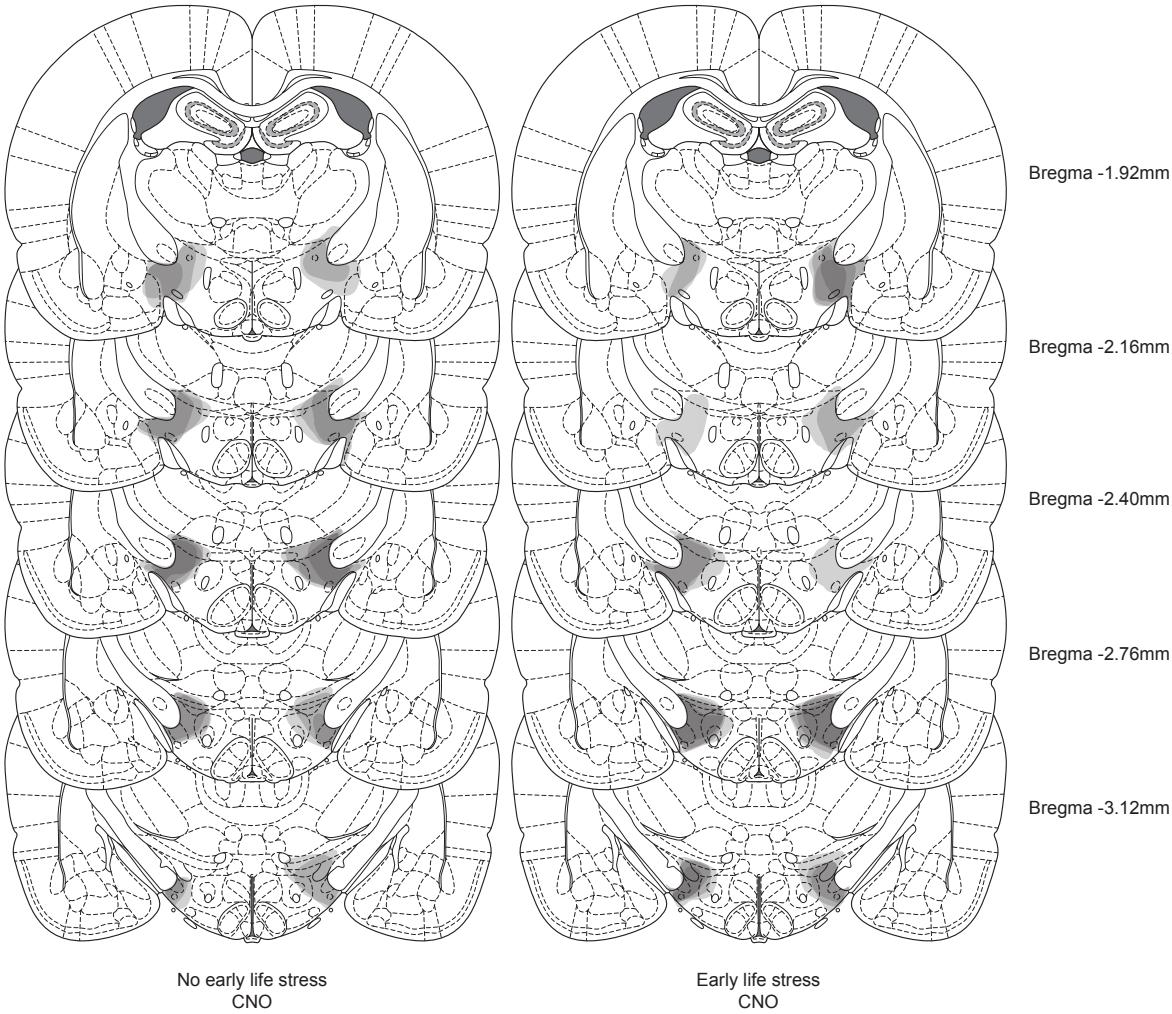


Figure S3. Clozapine-*N*-oxide (CNO)-treated rat viral expression. Extent of hM3D(Gq) viral expression in the lateral hypothalamus across the two CNO treatment groups. Minimum expression is represented by light grey and maximum expression is represented by dark grey. Coronal slices adapted from Paxinos & Watson[31].

CHAPTER FOUR

Recruitment of hypothalamic orexin neurons after formalin injections in adult male rats exposed to a neonatal immune challenge

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Author contributions to this manuscript

Erin J. Campbell	Designed and performed research, analyzed data and wrote the manuscript	
Stephanie M. Watters	Designed and performed research, analyzed data and wrote the manuscript	
Ihssane Zouikr	Designed and performed research, analysed data and wrote the manuscript	
Deborah M. Hodgson	Designed research and wrote the manuscript	
Christopher V. Dayas	Designed research, analyzed data and wrote the manuscript	

June, 2016

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Deputy Head of Faculty (Research and Research Training)

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Recruitment of hypothalamic orexin neurons after formalin injections in adult male rats exposed to a neonatal immune challenge

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Exposure to early life physiological stressors, such as infection, is thought to contribute to the onset of psychopathology in adulthood. In animal models, injections of the bacterial immune challenge, lipopolysaccharide (LPS), during the neonatal period has been shown to alter both neuroendocrine function and behavioral pain responses in adulthood. Interestingly, recent evidence suggests a role for the lateral hypothalamic peptide orexin in stress and nociceptive processing. However, whether neonatal LPS exposure affects the reactivity of the orexin system to formalin-induced inflammatory pain in later life remains to be determined. Male Wistar rats ($n = 13$) were exposed to either LPS or saline (0.05 mg/kg, i.p) on postnatal days (PND) 3 and 5. On PND 80–97, all rats were exposed to a subcutaneous hindpaw injection of 2.25% formalin. Following behavioral testing, animals were perfused and brains processed for Fos-protein and orexin immunohistochemistry. Rats treated with LPS during the neonatal period exhibited decreased licking behaviors during the interphase of the formalin test, the period typically associated with the active inhibition of pain, and increased grooming responses to formalin in adulthood. Interestingly, these behavioral changes were accompanied by an increase in the percentage of Fos-positive orexin cells in the dorsomedial and perifornical hypothalamus in LPS-exposed animals. Similar increases in Fos-protein were also observed in stress and pain sensitive brain regions that receive orexinergic inputs. These findings highlight a potential role for orexin in the behavioral responses to pain and provide further evidence that early life stress can prime the circuitry responsible for these responses in adulthood.

Keywords: orexin, hypocretin, pain, nociception, formalin, lipopolysaccharide, early life stress

INTRODUCTION

Chronic pain is a debilitating condition for which effective treatments and the underlying neurobiological mechanisms responsible are yet to be fully identified (Dersh et al., 2002). Pain characteristically evokes emotional responses in individual sufferers and is often comorbid with negative affective states including anxiety and depression and sleep disturbances (Neugebauer et al., 2004; Katon et al., 2007). Interestingly, the normal maturation of the nociceptive system is dependent on the uninterrupted development of sensory inputs in early life (Fitzgerald, 2005). Exposure to adverse events during this sensitive period of development, when the nociceptive circuitry is undergoing fine-tuning, can precipitate maladaptive pain processing in later life. For example, in humans, early life physical stressors have been associated with an increased risk for developing chronic pain conditions in adulthood (Davis et al., 2005). Similarly, recent preclinical research conducted by our group has indicated that early life exposure to the immune challenge lipopolysaccharide (LPS) resulted in hyperalgesia in response to formalin, a model of inflammatory pain, in young rats (Zouikr et al., 2014b). Despite recent efforts, the neural circuitry modulating the increased response to pain following early life stress remains to be fully determined.

Importantly, the hypothalamus controls neuroendocrine stress responses and nociceptive processing (Hsieh et al., 1996; Matthews, 2002). Direct afferent and efferent relays connect hypothalamic nuclei to brain areas involved in the active modulation of pain and nociception such as the dorsal horn of the spinal cord and the periaqueductal gray (PAG; Holland and Goadsby, 2007; Todd, 2010). Additionally, subregions such as the perifornical and lateral hypothalamic areas are key brain structures coordinating behavioral and autonomic stress responses and receive significant corticolimbic input (Millan, 2002). Recently, a subpopulation of hypothalamic neurons which produce the neuropeptides orexins (hypocretins), have been identified as possible substrates in the modulation of pain and stress through projections to the PAG, brainstem and paraventricular thalamus

(PVT; Peyron et al., 1998; Marcus et al., 2001). For example, injections of orexin-A into the rostral ventromedial medulla produced antinociceptive-like behavior in response to formalin in male rats (Azhdari-Zarmehri et al., 2014). Interestingly, maternal separation was found to alter orexin system reactivity to psychological stress in adulthood (James et al., 2014). Despite these results, only a few studies have assessed orexin system recruitment and also studied behavioral responses following noxious stimuli. Watanabe et al. (2005) demonstrated that prepro-orexin knockout mice exhibited increased nociceptive behaviors in response to peripheral inflammation and reduced stress-induced analgesia following footshock stress in adulthood compared to wild type mice. Further, Heidari-Oranjaghi et al. (2012) found that intracerebroventricular (i.c.v) injections of the OX_1 orexin receptor antagonist SB-334867, resulted in increased pain responses to formalin, but only following both restraint and swim stress. Recently, we demonstrated that rats exposed to neonatal LPS displayed enhanced formalin-induced flinching but not licking responses in adolescence at postnatal day (PND) 22 (Zouikr et al., 2014b). These behavioral changes were accompanied by attenuated Fos-protein cell counts in the rostral dorsal PAG as well as the rostral and caudal axes of the ventrolateral PAG. But, whether exposure to early life immune challenge alters orexin system function in response to a noxious stimulus in adulthood (PND 80–97) has not been determined. This information will improve our understanding as to how neonatal physical and emotional insults can rewire the brain pathways involved in pain and stress processing.

Therefore, the aim of the present study was to assess the effect of neonatal LPS exposure on the reactivity of the orexin system, as assessed by Fos-protein expression, to formalin challenge in adulthood (PND 80–97). Given the relationship between stress, nociception and the orexin system, it was hypothesized that rats exposed to LPS in early life would demonstrate increased pain and grooming responses to formalin in adulthood. We predicted that these behavioral responses would be accompanied by evidence of increased orexin cell activity, and that this recruitment pattern would also be reflected in Fos-responses in downstream projection targets of this system.

METHODS AND MATERIALS

ETHICS STATEMENT

All experiments performed were approved by the University of Newcastle Animal Care and Ethics Committee, and carried out in accordance with the National Health and Medical Research Council Australian Code of Practice for the care and use of animals for scientific practice.

ANIMALS

Four experimentally naïve female Wistar rats were obtained from the University of Newcastle Animal house and bred with two experimentally naïve males in the University of Newcastle vivarium. On PND 3 and 5 a random subset of animals from each litter were administered LPS as a neonatal immune challenge (detailed below). A total of 13 male offspring were included in this study, 6 LPS-exposed animals and 7 saline animals. On PND 21, animals were weaned with 2 animals/cage (41.5 ×28 × 22 cm cages; Mascot Wire Works, Sydney). Food (Rat and Mouse Pellets, Glen Forest, Western Australia) and water were available *ad libitum* and rats were maintained on a 12 h light (0600–1800): 12 h dark cycle. Temperature was maintained at 20 ± 2°C and humidity was kept at 34 ± 2%.

NEONATAL LPS CHALLENGE

The neonatal LPS procedure was performed as per previously published procedures (Walker et al., 2009). Between 0900 and 1000 h on PND 3 and 5 (birth as PND 1), pups in the LPS treatment condition were briefly removed from their home cages and administered 0.05 mg/kg LPS (intraperitoneally, i.p, LPS from *Salmonella enterica*, serotype *enteritidis*, Sigma-Aldrich, USA, dissolved in 20 µl sterile pyrogen-free saline). Saline controls were given an equal volume of sterile saline (Livingstone International, Australia). The timing of injection and dose were selected as it had been previously shown to produce a sustained immune response with no mortality (Walker et al., 2006).

FORMALIN TEST

This test has been previously described in our laboratory (Zouikr et al., 2014c) and is a well-validated model of behavioral responses to nociceptive stimuli (early phase or phase 1, first 5 min), inhibition of nociceptive responding (interphase, 5–15 min) and inflammation (late phase or phase 2, 15–60 min; Wheeler-Aceto and Cowan, 1991; Tjølsen et al., 1992; Henry et al., 1999; Fischer et al., 2014). Between PND 80 and 97 animals were removed from their home cage and a subcutaneous injection of formalin (2.25%) was administered into the plantar surface of the right hindpaw of all rats (50 µl formaldehyde 36.5–38%; Biolab Ltd, Victoria, Australia; sodium chloride injection BP 0.9% Pfizer, Australia). This volume and concentration of solution has been previously shown by our group to produce the biphasic response of the formalin test (Zouikr et al., 2013). A saline control injection into the hindpaw was not included, as this has been found to produce no pain-induced behaviors including licking and flinching (Guy and Abbott, 1992; Butkevich and Vershinina, 2001). The behavioral response to formalin was examined in transparent plexiglas boxes (30 × 30 × 30 cm) for 1 h. A researcher blind to experimental conditions scored behavioral responses using JWatcher ethograph software (version 0.9, Macquarie University, Sydney, Australia). Pain behaviors were measured by the number of flinches of the injected paw and the time spent licking the injected paw. Exploratory behavior was measured as the time spent rearing during the formalin test and grooming behavior during the formalin test was analyzed as the time spent grooming the forepaws.

IMMUNOHISTOCHEMISTRY

Ninety minutes following formalin injections, rats were deeply anaesthetized with an overdose of sodium pentobarbitone (200 mg/kg; i.p; Virbac, Australia) and transcardially perfused with 200 mL of 0.1 M phosphate buffered saline followed by 500 mL of 4% paraformaldehyde (pH 9.5). Brains were then removed and postfixed in 4% paraformaldehyde (24 h at 4°C) and then stored in 15% sucrose until sectioning. Serial coronal sections of the rostral forebrain (40-µm) and caudal midbrain (50-µm) were cut using a freezing microtome (Leica

Microsystems, SM2000R). A 1-in-4 series of sections from the hypothalamus (bregma −2.28 to −3.24), the PVT (bregma −2.28 to −3.24), the paraventricular nucleus of the hypothalamus (PVN; bregma −1.46 to −1.78) and the amygdala (bregma −2.28 to −3.08), and a 1-in-5 series of sections from the PAG (bregma −6.69 to −8.19) were processed for immunohistochemical detection of Fos-protein (72 h, 1:10000, rabbit polyclonal, sc-52, Santa Cruz Biotechnology, CA, USA) as described previously in detail (Dayas et al., 2008; James et al., 2014). Following primary antibody application, sections were incubated in a secondary antibody (2 h, 1:300, donkey anti rabbit, 711-065-152, Jackson IR, PA, USA). Hypothalamic sections were dual-labeled for orexin-A, also likely detecting pre-pro orexin (48 h, 1:15000, orexin-A antibody, goat polyclonal, sc-8070, Santa Cruz Biotechnology). The selectivity of this antibody has been illustrated in a recent study by Blanco-Centurion et al. (2013). Please see Supplementary Material S1 outlining the details of the specificity of the orexin-A antibody. Following orexin primary antibody application, sections were subsequently incubated in a secondary antibody (2 h, 1:400, donkey anti goat, 705-065-147, Jackson IR, PA, USA). An equal number of animals from each treatment group were included in each individual immunohistochemical run.

Bilateral counts of single-labeled Fos-positive cells were made in the PVT, PVN, basolateral amygdala (BLA), medial nucleus of the amygdala (MeA), the central nucleus of the amygdala including both lateral and medial subdivisions (CeL, CeM respectively), the dorsal PAG (including both the dorsomedial and dorsolateral columns), and the lateral and ventrolateral PAG using Metamorph Imaging System Software (Version 7.5; Molecular Devices Analytical Technologies) at 10x magnification (Olympus CX40). Quantification of Fos-positive cells was determined by creating a region of interest for each brain structure and a thresholding procedure was used to quantify Fos expression. Counts of Fos-positive orexin cells were made in the dorsomedial hypothalamus (DMH), the perifornical area (PFA), and the lateral hypothalamus (LH) by one observer, blind to treatment, using a 20x microscopic objective (Olympus CX40). The DMH was defined as the area between the third ventricle and the medial side of the PFA, the PFA was defined as the area surrounding the fornix and the LH was defined as the area from the lateral side of the PFA to the optic tract (Laorden et al., 2012; James et al., 2014). All brain coordinates were adapted from the Paxinos and Watson atlas (Paxinos and Watson, 2007).

DATA ANALYSIS

Initial analysis of covariance (ANCOVA) analyses revealed no significant effect of litter size on both behavioral and brain comparisons. Behavioral data was analyzed across neonatal treatment group using one-way between subjects ANOVAs for each phase of the formalin test. Using area under the curve calculations for the formalin test, phase 1 was considered the first 5 min, the interphase was the sum of 6–15 min, and phase 2 was the sum of responses from 16–60 min. Fos-protein immunohistochemical data was analyzed using two-way between subjects ANOVAs comparing neonatal treatment and brain region where appropriate, alternatively one-way ANOVAs were used. *Post-hoc* comparisons were assessed using least significant differences tests. Pearson's correlations were used to examine the relationship between the percentage of Fos-positive orexin cells in the subregions of the hypothalamus and behavioral responses of animals in phase 1 and the interphase of the formalin test. All statistical analyses were conducted using IBM SPSS V21 with an alpha value of 0.05. All figures are represented as means with standard errors.

RESULTS

EFFECT OF NEONATAL LPS EXPOSURE ON FORMALIN-INDUCED NOCICEPTIVE BEHAVIOR

One-way between subjects ANOVAs revealed no significant effect of neonatal treatment on flinching behavior in any phase of the formalin test (p's > 0.05; **Figures 1A,B**). An analysis of licking responses revealed a significant effect of neonatal treatment during the interphase with LPS animals displaying reduced time spent licking compared to saline controls [$F_{(1, 12)} = 3.795$, $p = 0.042$; **Figures 1C,D**].

EFFECT OF LPS ON FORMALIN-INDUCED EXPLORATORY AND GROOMING BEHAVIORS

One-way between subjects ANOVAs revealed a significant effect of neonatal treatment on the total time spent grooming in the interphase of the formalin test with LPS-treated animals spending more time grooming compared to saline animals [$F_{(1, 12)} = 6.96$, $p = 0.014$; **Figures 2A,B**]. LPS-treated animals also displayed significantly increased time grooming from phase 1 to the interphase of the formalin test compared to saline animals [$F_{(1, 12)} = 5.538$, $p = 0.022$; **Figure 2B**]. ANOVA also revealed no significant effects of neonatal treatment on time spent grooming during phase 1 or phase 2, nor for time spent rearing in any phase of the formalin test (p's > 0.05; **Figures 2C,D**).

EFFECT OF NEONATAL IMMUNE CHALLENGE ON OREXIN CELL REACTIVITY IN RESPONSE TO FORMALIN IN ADULTHOOD

There were no differences found in the total number of orexin-positive cells between LPS animals and saline controls in any subregion of the hypothalamus (p's > 0.05, **Table 1**). However, a two-way between subjects ANOVA revealed a significant main effect of neonatal treatment on the percentage of Fos-positive orexin cells with LPS-exposed rats exhibiting a greater percentage of Fos-positive orexin cells compared to saline-treated animals [$F_{(1, 246)} = 11.863$, $p = 0.001$, **Figure 3**]. Additionally, there was a significant interaction between neonatal treatment and hypothalamic subregion on the percentage of Fos-positive orexin cells [$F_{(2, 246)} = 3.387$, $p = 0.035$]. *Post-hoc* comparisons revealed that LPS animals displayed significantly greater percentages of Fos-positive orexin cells in the DMH and PFA compared to saline controls (p's < 0.05) however, no differences were found in the LH ($p > 0.05$, **Figure 3**).

EFFECT OF LPS ON FOS-PROTEIN EXPRESSION IN THE PVT, PVN, AMYGDALA AND PAG

A one-way between subjects ANOVA revealed that LPS-treated animals exhibited significantly greater numbers of Fos-positive cells in the PVT and PVN compared to saline controls [$F_{(1, 82)} = 59.055$, $p < 0.001$; $F_{(1, 31)} = 9.370$, $p = 0.005$; **Figures 4A,B**].

A two-way between subjects ANOVA revealed a significant interaction of neonatal treatment and amygdala subregion on the

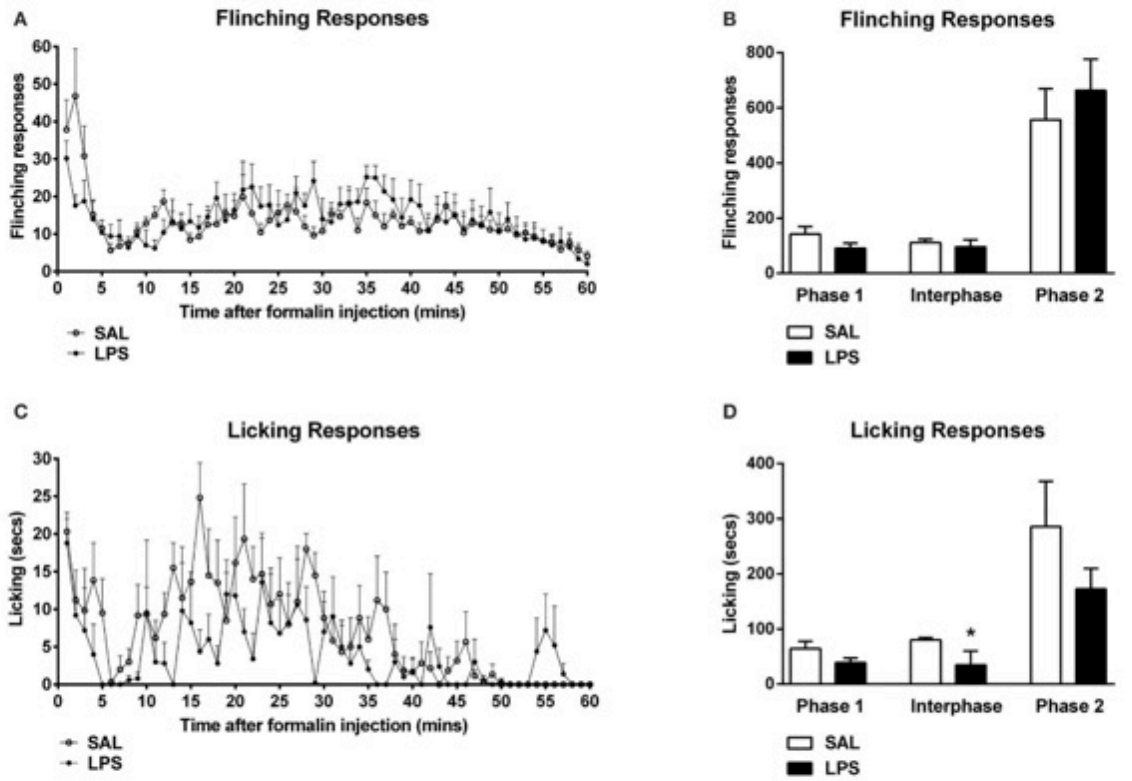

FIGURE 1 | Neonatal LPS exposure enhanced pain suppression of licking behaviors during the interphase in response to formalin in adulthood. Time course of flinching and licking responses for LPS and saline rats exposed to formalin in adulthood **(A,C)**. No differences were observed in flinching behavior across any phase of the formalin test between LPS-treated rats and saline controls **(B)**. LPS-exposed rats exhibited a potentiated inhibitory pain response to formalin in licking behaviors during the interphase, with no effect of neonatal treatment on phase 1 or phase 2 of the formalin test **(D)**. Data are presented as mean + standard error. $^*p < 0.05$. SAL: $n = 7$; LPS: $n = 6$.

number of Fos-positive cells [$F_{(3, 280)} = 2.896$, $p = 0.036$]. Least significant differences comparisons revealed that LPS-treated animals displayed a significantly greater number of Fos-positive cells in the MeA compared to saline animals ($p = 0.001$; **Figure 4C**).

A two-way between subjects ANOVA revealed a main effect of neonatal treatment on the number of Fos-positive cells within the PAG [$F_{(1, 204)} = 38.440$, $p < 0.001$]. *Post-hoc* comparisons revealed that LPS-treated animals displayed significantly greater numbers of Fos-positive cells in the dorsal ($p = 0.022$), lateral ($p < 0.001$) and ventrolateral PAG ($p < 0.001$) compared to saline animals (**Figure 4D**).

CORRELATIONS BETWEEN OREXIN CELL ACTIVITY AND BEHAVIORAL RESPONSES TO FORMALIN IN ADULTHOOD

Correlation analyses revealed a negative correlation between the total time spent licking in phase 1 of the formalin test and the percentage of Fos-positive orexin cells in the DMH ($r = -0.806$, $p = 0.005$) and the PFA ($r = -0.691$, $p = 0.027$; **Figures 5A,B**). In the interphase of the formalin test, total time spent grooming was also positively correlated with the percentage of Fos-positive orexin cells in the DMH ($r = 0.634$, $p = 0.049$) and PFA ($r = 0.673$, $p = 0.033$, **Figures 5C,D**).

DISCUSSION

In the current study we show that animals exposed to LPS in early life exhibit altered behavioral responses to a formalin challenge in adulthood. LPS-treated animals displayed increased orexin cell activity, as assessed by Fos-like immunoreactivity in the DMH and PFA but not LH. Additionally, increases in numbers of Fos-positive neurons were observed in stress and pain sensitive brain regions that express orexin receptors including the PVT, PVN, MeA, and PAG.

INCREASED OREXIN CELL REACTIVITY TO FORMALIN FOLLOWING AN EARLY LIFE IMMUNE CHALLENGE

The primary aim of this study was to examine the response of orexin neurons to an acute formalin injection in adulthood following early life LPS exposure. Using Fos-protein immunohistochemistry, we observed an increase in the recruitment of DMH and PFA orexin neurons in LPS-exposed rats compared to controls. Interestingly, no change in orexin cell reactivity to formalin was observed in the LH. This differential recruitment pattern is interesting given the recent suggestions of a dichotomy of function between medial and lateral orexin cell populations (Estabrooke et al., 2001; Harris and Aston-Jones, 2006). For

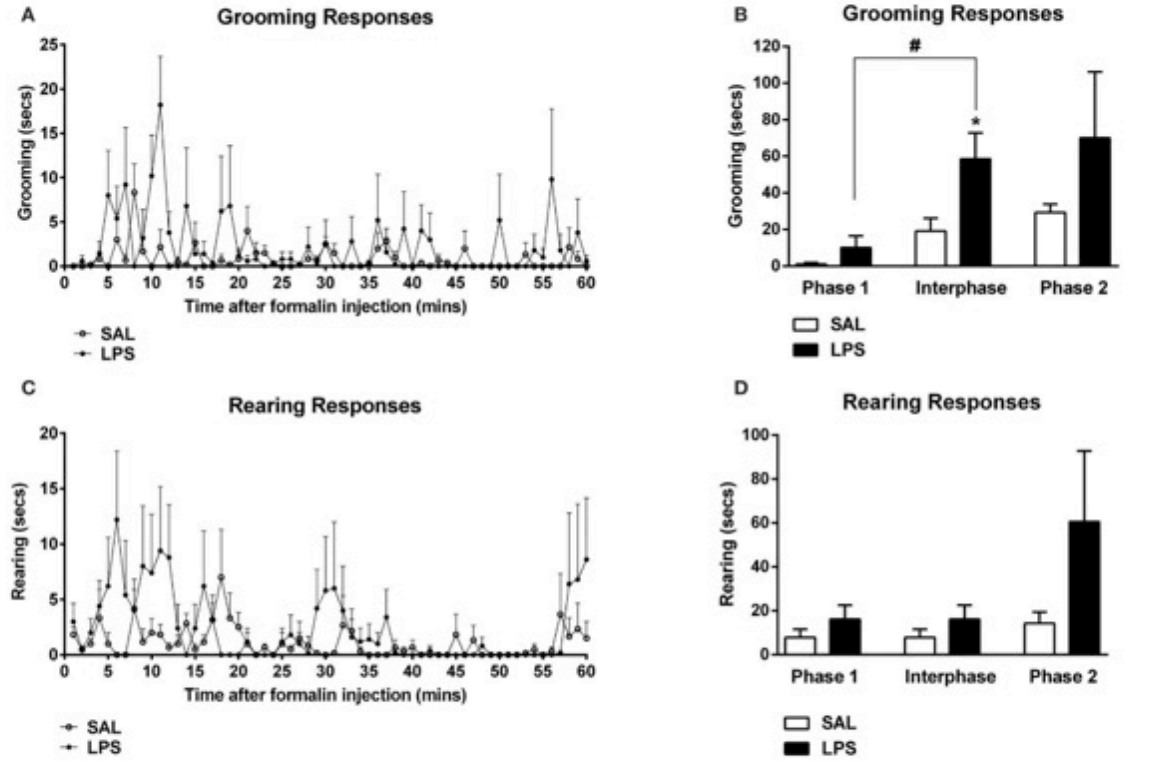

FIGURE 2 | An early life immune challenge increased self-grooming responses to formalin in adulthood. Time course of grooming and rearing responses for LPS and saline animals **(A,C)**. LPS-treated rats displayed increased grooming behaviors in response to formalin when compared to saline animals **(B)**. There was no effect of neonatal treatment on rearing behavior across all phases of the formalin test **(D)**. Data are presented as mean + standard error. $*p < 0.05$, $\#p < 0.05$ phase 1 vs. interphase. SAL: $n = 7$; LPS: $n = 6$.

Table 1 | Orexin cell expression in each subregion of the hypothalamus.

Treatment	Region	Orexin number	Fos-Orexin number
Saline ($n = 6$)	DMH	30.77 ± 3.65	11.97 ± 1.55
	PFA	111.33 ± 10.56	19.99 ± 2.71
	LH	35.18 ± 2.33	3.35 ± 0.41
LPS ($n = 6$)	DMH	30.89 ± 2.85	16.89 ± 2.34
	PFA	101.59 ± 7.02	29.62 ± 3.77
	LH	39.60 ± 3.13	4.72 ± 0.63

Data presented as average number of orexin cells or Fos-positive orexin cells ± standard error mean in the dorsomedial hypothalamus (DMH), perifornical area (PFA), and lateral hypothalamus (LH).

example, orexin neurons in the DMH and PFA have been linked with arousal and the modulation of the stress response whereas those located in the LH have been linked with reward (Harris et al., 2005). In support, rats administered sodium-lactate to induce panic anxiety exhibited increased activation of DMH-PFA but not LH orexin neurons (Johnson et al., 2010). Thus, arousal and stress responsive orexin cells may be preferentially sensitized by early life immune stress.

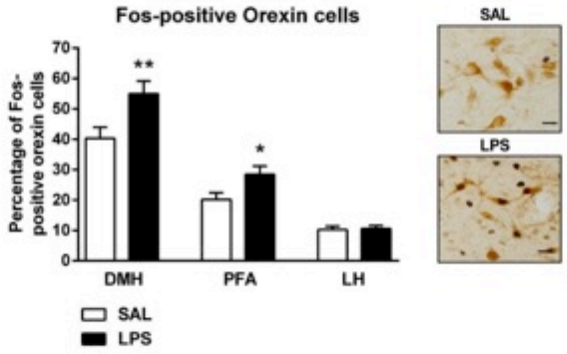

FIGURE 3 | LPS resulted in an increase in the percentage of Fos-positive orexin cells in both the dorsomedial (DMH) and perifornical (PFA) subregions of the hypothalamus. The percentage of Fos-positive orexin cells in the DMH and PFA was significantly higher in LPS-exposed animals compared to saline controls. No differences were observed in orexin cell activity in the lateral hypothalamus (LH) across neonatal treatment groups. Photomicrographs of coronal sections of the hypothalamus immunolabeled for Fos-protein and orexin. Data are presented as mean + standard error. $*p < 0.05$, $**p < 0.01$, scale bar 20 μm. SAL: $n = 6$; LPS: $n = 6$.

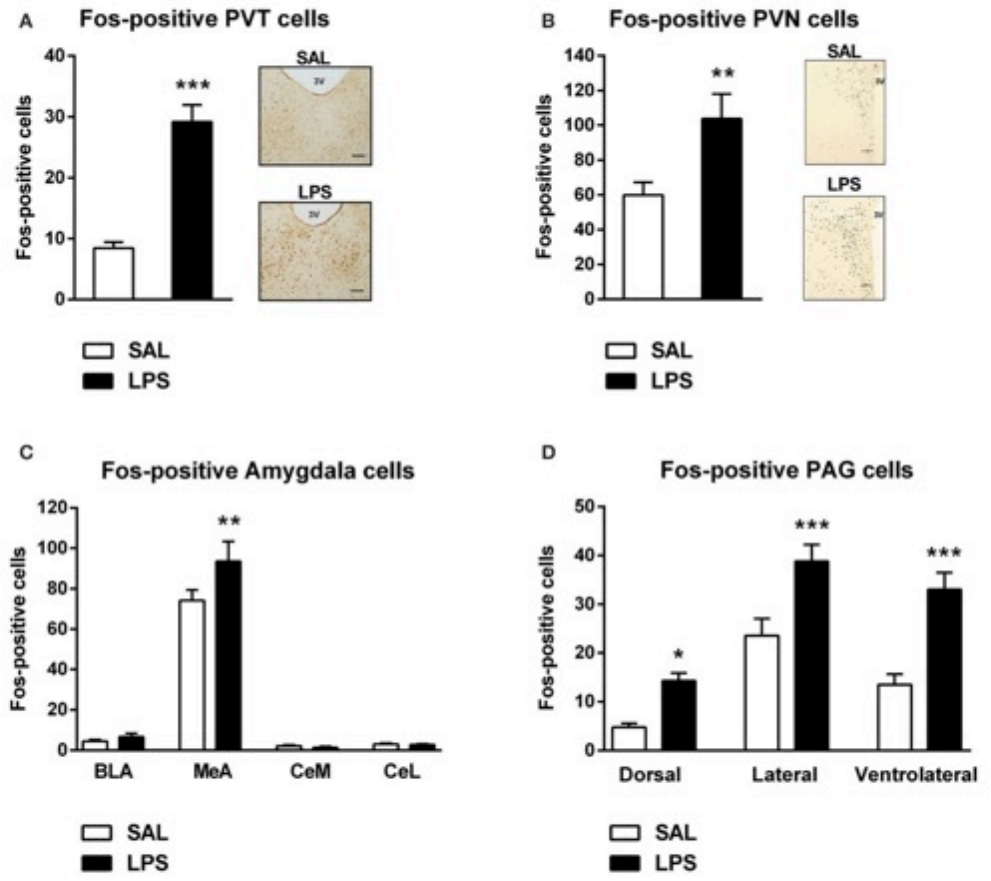

FIGURE 4 | Neonatal LPS treatment resulted in an increase in the number of Fos-positive cells in the paraventricular thalamus (PVT), paraventricular nucleus (PVN), the medial nucleus of the amygdala (MeA) and the periaqueductal gray (PAG). Early life LPS treatment resulted in an increase in Fos activity in both the PVT and PVN **(A,B)**. Photomicrographs of coronal sections of the PVT and PVN immunolabeled for Fos-protein **(A,B)**. LPS-treated animals also exhibited an increased number of Fos-positive cells in the MeA **(C)**. There was no effect of neonatal treatment on any other subregion of the amygdala. LPS-exposed rats also had increased Fos-protein expression in the dorsal, lateral and ventrolateral PAG **(D)**. Data are presented as mean + standard error. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, scale bar 100 μm, 3V = third ventricle. SAL: $n = 6$; LPS: $n = 6$.

It is unclear from the present study which pain sensitive afferent pathways may have been involved in the recruitment of orexin neurons. Brain sites that receive direct afferent inputs from lamina I projection neurons in the superficial dorsal horn include the ventrolateral medulla, nucleus of the solitary tract, lateral parabrachial nucleus, PAG and the thalamus (Millan, 2002; Gauriau and Bernard, 2004; Todd, 2010). The parabrachial nucleus is interesting in this regard possessing efferent projections to the amygdala and the LH (Bernard et al., 1993; Bester et al., 1997; Gauriau and Bernard, 2002). These inputs may directly recruit orexin neurons. Additionally, the spinal cord sends direct projections to the hypothalamus. For example, Burstein et al. (1987) demonstrated direct projections from three regions of the spinal gray matter, including the lateral reticulated area, the area surrounding the central canal and the marginal zone, to the hypothalamus (Burstein et al., 1987; Giesler, 1995). However, the direct input from these spinal cord regions to orexin neurons is yet to be examined. Interestingly, the stress responsive central nucleus of the amygdala (CeA) and MeA also project to the LH (Peyron et al., 1998; Yoshida et al., 2006) and may provide "top-down" afferent input to the LH. Clearly, further work will be required to determine whether the activation of orexin neurons occurs through ascending nociceptive pathways or descending inputs from stress responsive centers such as the amygdala. It is important to acknowledge that because previous work has shown that hindpaw saline injections cause no pain-induced behaviors including licking and flinching (Guy and Abbott, 1992; Butkevich and Vershinina, 2001), we did not include a Fos control group for the formalin challenge. While it is difficult to determine the direction of change from baseline in our Fos-induced orexin cell reactivity, prior studies have shown that rats subjected to hindpaw saline injections showed no Fos labeling in the lumbar spinal cord (Yi and Barr, 1995). Additionally, Barr (2011) reported that 14 day old rat pups given saline into their hindpaw did not exhibit Fos labeling in the PVN or dorsal/lateral PAG.

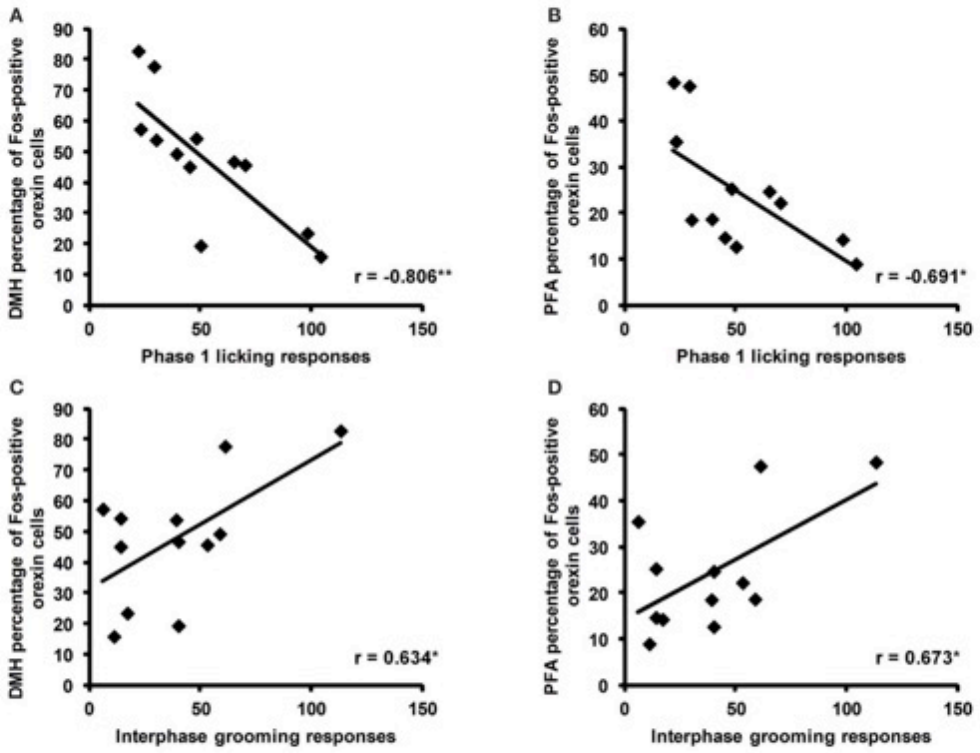

FIGURE 5 | Correlations of the percentage of Fos-positive orexin cells with licking and grooming behaviors during the formalin test. Significant negative correlations were found between the time spent licking (secs) during phase 1 of the formalin test and the percentage of Fos-positive cells in the dorsomedial hypothalamus (DMH) and perifornical area (PFA; **A,B**). Significant positive correlations were found between the time spent grooming (secs) during the interphase of the formalin test and the percentage of Fos-positive orexin cells in the DMH and PFA **(C,D)**. $^{*}p < 0.05$, $^{**}p < 0.01$. SAL: $n = 6$, LPS: $n = 6$.

ENHANCED FORMALIN-INDUCED INHIBITORY PAIN RESPONSE IN LPS-TREATED RATS

The formalin test is a well-established animal model of persistent pain (Tjølsen et al., 1992). Three distinct behavioral responses are commonly associated with the formalin test. The early phase, or phase 1, involves the direct chemical stimulation of nociceptors, the interphase denotes the active inhibition of pain and the late phase, or phase 2, represents the inflammatory pain response (Dubuisson and Dennis, 1977; Tjølsen et al., 1992; Franklin and Abbott, 1993; Henry et al., 1999; Fischer et al., 2014). Importantly, of these three phases, the interphase has received the least attention. Here, we demonstrated decreased licking behaviors in the interphase after formalin challenge in LPS animals. This is perhaps not surprising given that increased grooming may override or mask changes in licking behavior. Although a slight trend was observed in phase 2 we found no significant changes in flinching behavior in response to formalin between treatment groups in male rats. This is in contrast with our recently published work whereby adult rats exposed to a neonatal LPS challenge displayed a significantly increased flinching response during the late phase of the formalin test (Zouikr et al., 2014a). This discrepancy could be attributed to methodological differences, presently, we analyzed the interphase whereas Zouikr et al. (2014a) focused on phase 2 of the formalin test. Further, differences in behavioral scoring methods (manual vs. software) may have contributed to this discrepancy. Lastly, enhanced grooming in the current cohort may have masked changes in flinching. The putative enhancement of pain suppression observed in the interphase of LPS-treated rats is interesting given the increase in orexin cell reactivity in LPS-exposed rats. Furthermore, overall licking behaviors of LPS-treated rats negatively correlated with the percentage of Fos-positive orexin cells in the DMH and PFA. These findings are consistent with data implicating the orexin system in descending inhibitory pain pathway control (Bingham et al., 2001).

In the current study we also observed an increase in Fos-protein in all subregions of the PAG in neonatally LPS-treated rats compared to controls. It is important to note that the PAG is anatomically organized into longitudinal columns including the dorsal PAG, the lateral PAG and the ventrolateral PAG and each column plays distinct roles in the response to both stress and pain (Bandler and Shipley, 1994). The PAG has a well-characterized role in analgesia and stress coping and is the recipient of orexinergic innervation (Peyron et al., 1998; Keay and Bandler, 2002; Gebhart, 2004; Chapman et al., 2008). It is possible that the increase in orexin cell activity and the enhanced inhibitory pain response to formalin contribute to the active inhibition of pain through projections to the PAG. In support, Azhdari-Zarmehri et al. (2011) found that microinjections of orexin-A into the

PAG enhanced inhibitory pain responses in the interphase in response to formalin in adulthood. Indeed, orexin-A has been shown to reduce inhibitory postsynaptic currents in ventrolateral PAG neurons that directly project to the rostral ventromedulla (Ho et al., 2011). Of the PAG columns, the lateral PAG tends to receive stronger orexin inputs, which is interesting given its role in active coping strategies in the response to pain (Bandler and Shipley, 1994; Yoshida et al., 2006). Accordingly, the pattern of Fos activity we observed in the PAG may reflect affective coping strategies in response to formalin-evoked stress. For example, Keay and Bandler (2001) demonstrated that increased Fos activity in both the ventrolateral and lateral PAG is linked with altered emotional coping responses to persistent pain. These results may help explain the affective behavioral changes observed in response to formalin as described in more detail below.

EARLY LIFE LPS EVOKED AN AFFECTIVE-LIKE BEHAVIORAL RESPONSE TO FORMALIN

We also examined the affective-like responses to formalin in adulthood by assessing both grooming and rearing. Self-grooming is thought to reflect a coping mechanism to produce de-arousal (Spruijt et al., 1992; Kalueff and Tuohimaa, 2005; Lariviere et al., 2011). In our study, LPS-exposed animals spent significantly more time grooming in response to formalin in adulthood compared to saline controls. No differences were observed in rearing behavior. In keeping with our findings, Aloisi et al. (1998) demonstrated that exposure to acute restraint stress in adulthood increased self-grooming during the interphase of the formalin test. Interestingly, our study found that an increase in orexinergic activity was correlated with an increase in grooming behavior following formalin injection. These data are in line with previous research implicating dysregulated orexin system function in affective behavioral responses to stress or adverse experiences in adulthood (Johnson et al., 2010; Li et al., 2010; James et al., 2014; Yeoh et al., 2014). Further, Low and Fitzgerald (2012) have demonstrated an increase in the number of Fos-positive orexin cells in animals exposed to neonatal pain followed by later life pain. Low and Fitzgerald (2012) also found this orexinergic activity to be negatively correlated with rearing behavior. Together these results suggest that the orexin system may be susceptible to early life immune or emotional challenges, which promote neuroadaptations that manifest as dysregulated behavioral and neural responses to painful stimuli in later life.

FOS-PROTEIN EXPRESSION IN STRESS RESPONSIVE BRAIN REGIONS

We identified an increased pattern of Fos-protein in brain regions that are known to receive strong orexinergic input and are involved in the neuroendocrine and behavioral response to stress or pain modulation (Peyron et al., 1998; Marcus et al., 2001; Vanegas and Schaible, 2004). The brain regions we examined were the PVT, PVN, amygdala and PAG. LPS-exposed animals displayed increased Fos-protein expression in the PVT and PVN. The PVN and PVT both play an important role in the neuroendocrine and autonomic responses to stress. PVN corticotrophin-releasing factor cells constitute the apex of the hypothalamic-pituitary-adrenal (HPA) axis and the PVT is involved in regulating the HPA axis response to chronic stressors (Bhatnagar and Dallman, 1998; Dayas et al., 2004; Kirouac et al., 2005). Increased Fos immunoreactivity in the PVN and PVT is therefore consistent with other studies demonstrating activation of these stress response systems to a variety of physical stressors including painful stimuli such as cold and formalin-induced pain (Pacák and Palkovits, 2001).

Surprisingly, we found no changes in the numbers of Fos-positive CeA nuclei between treatment groups. This result contrasts previous research demonstrating increased CeA activity in response to physical stressors including persistent pain and acute pain or stress (Dayas et al., 2001; Neugebauer et al., 2004). Notably, orexin-immunoreactive fibers and orexin receptors are also observed in other subregions of the amygdala including the MeA (Peyron et al., 1998; Marcus et al., 2001). The MeA is typically sensitive to psychological stressors and has recently been identified as a central site mediating repetitive self-grooming behaviors, which has linked it to a range of neuropsychiatric disorders (Dayas et al., 1999, 2001; LeDoux, 2000; Hong et al., 2014). Interestingly, we observed a significant increase in Fos-positive nuclei in the MeA of LPS-exposed animals, an effect associated with elevated self-grooming responses to formalin. It is possible that ascending spinoparabrachial projections may recruit an orexin → MeA pathway resulting in affective-like behavioral responses to formalin. However, the MeA also provides input to DMH/PFA and may be recruited by ascending pain sensitive-pathways. Further work will be required to understand the hierarchical sequence for how these brain regions are recruited by formalin.

Taken together, the behavioral data presented here confirmed that animals exposed to an early life immune challenge exhibited an enhanced inhibition of pain during the interphase. These changes were associated with increased grooming behavior, which was strongly correlated with numbers of Fos-positive orexin neurons in the DMH/PFA. Our results are interesting given evidence that patients suffering from chronic pain disorders tend to suffer more from the affective disturbances of pain than frank pain itself (Crombez et al., 1999). Further, processes modulated by the orexin system eg. sleep, feeding, and motivation, are often disturbed in people suffering chronic pain states (Dersh et al., 2002). Thus, pharmacological or non-pharmacological interventions that restore normal orexin system function may prove beneficial in the treatment of chronic pain states.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fnins.2015.00065/abstract

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